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Regulation of RNA polymerase III transcription by TATA binding protein-associated CK2

by

© Ataollah Ghavidel

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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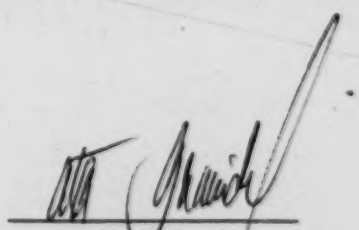

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ABSTRACT

Transcription by RNA polymerase III (pol III) encodes a variety of small RNAs that function in protein translation and RNA processing. Pol III transcription is strictly regulated throughout the cell cycle, according to cellular growth rate, and in response to diverse external cues. It is shown here that in *Saccharomyces cerevisiae*, a subset of oncogenic casein kinase 2 (CK2) is physically and functionally associated with the pol III core transcription factor TFIIIB. CK2 activates TFIIIB DNA binding and, in doing so, promotes pol III transcription. This effect is likely mediated by direct phosphorylation of the TBP subunit of TFIIIB by CK2.

In response to DNA damage, cells elicit a variety of physiological responses that collectively enhance cellular viability and maintain genomic integrity. These include induction of cell cycle arrest, upregulation of genes involved in DNA repair, and inhibition of DNA replication. It is demonstrated here that repression of pol III transcription is a novel cellular response to DNA damage. This transcriptional response is mediated via regulation of the TBP-associated CK2. Thus the targeted dissociation of CK2 catalytic subunits from TBP results in transcriptionally impaired TFIIIB. The resultant reduction in pol III transcription may promote cell cycle arrest and enhance cellular homeostasis in cells with DNA damage.

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ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CIP	calf intestine alkaline phosphatase
CK2	casein kinase 2
DNA	deoxyribose nucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl) ether
GST	glutathione-S-transferase
GTP	guanosine triphosphate
Ig	immunoglobulin
kan^r	kanamycin-resistance
MMS	methyl methanesulfonate
PAGE	polyacrylamide gel electrophoresis
PAP	potato acid phosphatase
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA buffer
TBP	TATA-binding protein
YDBI	yeast dialysis buffer I

Chapter 1

General introduction

In eukaryotes, nuclear transcription is carried out by three distinct RNA polymerases, namely, RNA polymerase (pol) I, II, and III. Each polymerase is dedicated to the transcription of different sets of genes that contain different promoter elements (reviewed in Hernandez 1993; Lee and Young 1998). These basal promoters are sufficient to determine RNA polymerase specificity. The polymerases do not recognize their target promoters directly. Instead, the promoters are first recognized by promoter-specific basal transcription factors which recruit the corresponding RNA polymerase by interacting with the distinct subunits of each polymerase.

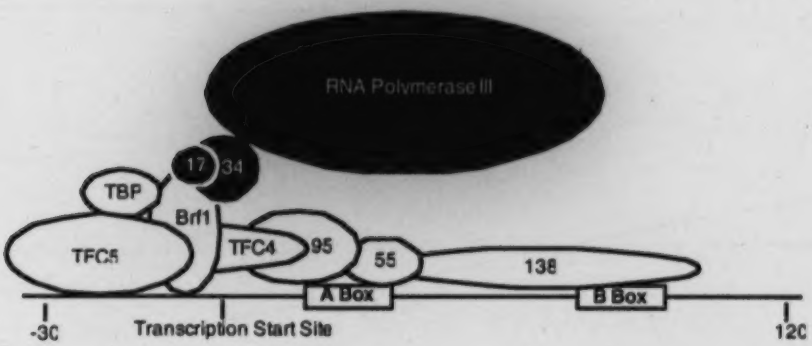
Transcription by RNA polymerase I and II, encoding the RNA components of the ribosomes and the mRNAs, respectively, will be discussed later. RNA polymerase III transcription in *Saccharomyces cerevisiae* constitutes the focal point of this study and will be described accordingly.

The General features of RNA polymerase III transcription

The genes transcribed by RNA polymerase III are functionally diverse (reviewed in Paule and White 2000; Huang and Maraia 2001). These include small untranslated tRNAs and the 5S RNA subunit of the ribosomes that participate in translation. Polymerase III additionally transcribes several other small RNAs that function as components of the RNA processing machinery. These include U6 small

nuclear RNA that functions in pre-mRNA splicing, and Rnase P RNA that is required for 5' end maturation of pre-tRNAs.

The core transcription machinery of RNA polymerase III consists of transcription factor IIIC (TFIIIC), transcription factor IIIB (TFIIIB), and the RNA polymerase III enzyme (Kassavetis et al. 1999). With few exceptions, pol III promoters are located within the transcribed DNA (Kassavetis et al. 2001). A schematic of a yeast tRNA gene transcription is depicted below:



Binding of TFIIIC (depicted in gray) to the A Box and B Box promoter elements is the initial step in the assembly of the pre-initiation complex. These elements, typically located 20 and 80 nucleotides downstream from the transcription start site, respectively, are 10-12 bp long nearly half of which are invariant among tRNA genes (Kassavetis et al. 1992; Geiduschek and Kassavetis 1995). In *S. cerevisiae*, TFIIIC is comprised of six polypeptides ranging in size from 55 to 138 kDa (Kassavetis et al. 1995). The genes encoding the three largest subunits are essential (Geiduschek and Kassavetis 1995).

The insight into TFIIIC-tDNA complex comes largely from the localization of the various subunits along the tDNA by site-specific protein DNA cross linking (Dumay et al. 1999; Geiduschek and Kassavetis 2001). The 138 kDa subunit binds to

the B Box, whereas, the 95 and 55 kDa subunits are cross linked to the A Box region. The simultaneous binding of TFIIIC to these regions is remarkable given that the separation between the A Box and B Box varies substantially among different tRNA genes in order to accommodate the range of variable stem regions found in eukaryotic tRNAs. Furthermore, the relative helical orientation of the A Box and B Box does not affect transcription (Bartholomew et al. 1993). The molecular mechanism underlying this elasticity is not clear, but electron microscopy analysis suggests that TFIIIC is flexible and can stretch in order to accommodate the sequence diversity among different pol III promoters (Bartholomew et al. 1993; Bartholomew et al. 1994).

The principal function of TFIIIC in pol III transcription is to recruit TFIIIB. This is accomplished by its second largest subunit of 120 kDa (Tfc4) which extends to the upstream region and positions TFIIIB at or near 30 base pairs upstream from the initiation site. TFIIIC-directed positioning of TFIIIB might explain why: 1) there is no obvious sequence conservation in the upstream region of the tRNA genes, and 2) in contrast to the deleterious effects of point mutations within either A Box or B Box, sequence substitutions within this region are well tolerated (Dumay et al. 1999; Flores et al. 1999).

In *S. cerevisiae*, TFIIIB (subunits depicted in white) is composed of TATA binding protein (TBP) and its associated 70 and 90 kDa subunits, all of which are essential (Geiduschek et al. 1995). TBP is shared among pol I, II, and III machineries and, with very few exceptions (Usheva and Shenk 1994; Hansen et al. 1997), is required for all cellular transcription (Cormack and Struhl 1992; Schultz et al. 1992). The 70 kDa subunit, termed Brf1 (TFIIB-Related Factor), displays 44% similarity to pol II transcription factor TFIIB, and similarly binds to TBP. The homolog of Brf1 in human has been identified and exhibits 41% identity to its yeast counterpart (Yieh et al. 2000). The 90 kDa Tfc5 displays no significant homology to other known proteins. It is likely that there exists a human homolog of Tfc5 since yeast Tfc5 reconstitutes transcription in vitro in combination with recombinant human TBP and Brf1 (Teichmann et al. 1997).

Most pol III transcribed genes lack a TATA sequence, so TBP containing TFIIIB can not directly bind these promoters, thereby necessitating its recruitment by DNA-bound TFIIIC (Huet and Sentenac 1992). The recruitment of TFIIIB to the

region upstream of the initiation site is mediated by interaction of its Brf1 subunit with Tfc4 of the DNA-bound TFIIIC. Once recruited, yeast TFIIIB footprints nearly 40 bp of the upstream region due to the simultaneous binding of all its subunits to DNA (Kassavetis et al. 1992; Kassavetis et al. 1998). Binding of TFIIIB to upstream DNA, although not sequence specific, is extremely stable; yeast TFIIIB remains DNA-bound in >1M KCl despite the fact that it alone does not recognize a TATA-less promoter (Kassavetis et al. 1990). It is postulated that interaction with TFIIIC unmasks a latent DNA-binding domain that allows for stable binding of TFIIIB to DNA (Kassavetis et al. 1990). Indeed the carboxyl-terminus of Brf1 binds DNA in a sequence-independent manner, whereas, full length protein does not (Huet et al. 1996).

The amino terminal of TBP varies in length, shows almost no sequence conservation among different organisms, and is largely dispensable for basal transcription in yeast (Cormack et al. 1991; Reddy and Hahn 1991; Cormack et al. 1994). On the other hand, the 180 amino acid carboxy-terminal segment is phylogenetically conserved and highly sensitive to mutations. This region contains two direct repeats that flank a highly basic segment. The crystal structure of DNA-bound yeast TBP reveals a highly symmetric DNA binding by two topologically identical domains that are derived from the two direct repeats (Fig. 1-1). The intramolecular symmetry generates a saddle-shaped structure in which the concave face interacts with the minor groove of DNA, while the solvent exposed highly basic convex face may provide binding surface for other molecules.

DNA photoaffinity labeling experiments of DNA-bound yeast TFIIIB indicate that the TBP subunit is in close proximity to the minor groove of the TATA-like DNA sequence which starts 30 nucleotides upstream of the start site of the $tRNA^{Tyr}$ gene (Persinger et al. 1999; Persinger and Bartholomew 2001). These experiments and the mutational analysis of TBP (Colbert et al. 1998; Kassavetis et al. 1998) indicate that interactions of TBP with DNA in a TFIIIB-DNA complex are similar to those of TBP bound to a TATA box, where it primarily contacts the minor groove of DNA. Furthermore, the available data suggest that DNA-bound Brf1 and Tfc5 subunits of TFIIIB form a protein clamp by binding to opposite sides of the TBP-DNA complex.

This structural organization may explain the unusual stability of the TFIIIB-DNA complex (Kassavetis et al. 1992).

The structure of the canonical TBP-TATA complex demonstrates that TBP induces a sharp bend in the eight base pairs of DNA centered around the TATA box (Fig. 1-1). This produces an underwound shallow minor groove that fits with the concave surface of the TBP saddle. It is proposed that the bend in DNA may bring the components of the transcription initiation machinery into proper apposition (Kim et al. 1993; Steitz 1990). This bend in DNA is maintained, and in fact exaggerated, after binding of TFIIIB (Colbert et al. 1998). This may provide a structural basis for the observed interaction between Brf1 and Tfc5 while they are bound to the opposing sides of the TBP-DNA complex (Kassavetis et al. 1998). Once DNA is bound, TFIIIB recruits polymerase to the promoter and positions it over the initiation site. All three subunits of TFIIIB are required for pol III recruitment, but a direct interaction with the polymerase has only been demonstrated for Brf1 (Kassavetis et al. 1997).

Yeast RNA polymerase III (depicted in black) comprises 15 characterized subunits all of which are essential for cell viability (Huang and Maraia 2001). While five of the subunits are shared by all three RNA polymerases, it is the interaction of pol III-specific 17 and 34 kDa subunits with Brf1 that mediates polymerase recruitment (Huet et al. 1996). Following polymerase recruitment, the double stranded DNA is melted around the initiation site to allow polymerase access to the template strand (Kassavetis et al. 1990; Kassavetis et al. 1992). This process requires the participation of TFIIIB, as certain mutations in Brf1 or Tfc5 allow proper polymerase positioning, but prevent the formation of an open promoter complex (Kassavetis et al. 1998). Surprisingly, TFIIIC remains bound to the internal promoter even after multiple passages of the polymerase (Braun et al. 1992; Kassavetis et al. 2000). Moreover, removing TFIIIC from a pol III template does not alter the rate of polymerase elongation in vitro (Matsuzaki et al. 1994). It is proposed that pol III transiently displaces TFIIIC subunit(s) as it transcribes through, while the TFIIIC complex as a whole remains associated with DNA due to its other DNA-bound subunits that are not being transcribed through (Paule and White 2000).

In contrast to pol I and pol II, yeast pol III recognizes a termination signal, a cluster of four or more T residues, accurately and efficiently without a requirement for termination factors (Andrau et al. 1999). After the initial round of transcription, TFIIIB can direct subsequent rounds of transcription considerably faster. Template commitment assays indicate that pol III is recycled after termination without being released from the template (Dieci et al. 1995). This arrangement produces a high density of transcribing polymerases on pol III transcribed genes. The procedural efficiency of pol III transcription, along with the dispersion of pol III genes in multiple copies throughout the genome, results in a level of transcription that combined with pol I transcription accounts for more than 80% of total cellular RNA synthesis in yeast (Warner 1999).

Human homologs with high degrees of sequence similarity to that of nearly all pol III factors characterized in *S. cerevisiae* have been identified and some have been functionally characterized (Kumar et al. 1998, Moir et al. 2000, also reviewed in Huang and Maria, 2001). Moreover, the promoter organization of pol III genes is remarkably similar among eukaryotes (Huang and Maraia 2001). It is generally believed that pol III transcription is mechanistically conserved throughout eukaryotes (White et al. 1995; Teichmann et al. 1997; Scott et al. 2001).

The General features of protein casein kinase 2 (CK2)

Protein kinase CK2 is a conserved and ubiquitously expressed dual specificity kinase. It is typically composed of two subunits, α and β, that combine to form the tetrameric α2β2 holoenzyme (Pinna and Meggio 1997; Glover 1998). In most eukaryotes there are two homologous but distinct α and α' catalytic subunits. The catalytic subunits from different species exhibit a high degree of homology; amino acid identity is 57% between *S. cerevisiae* and *Drosophila* and 90% between *Drosophila* and human (Guerra et al. 1999). With the exception of *S. cerevisiae* and *A. thaliana* (each with two distinct β regulatory subunits), there is a single β isoform in eukaryotes (Glover 1998).

The catalytic subunits of CK2 either alone or within the tetrameric complex are constitutively active (Guerra et al. 1999). The crystal structure of recombinant maize CK2α provides a rationale for the constitutive activity of the enzyme in vitro

(Fig. 1-2). The N-terminal segment of the kinase interacts with the activation domain which spans residues 175-201 and neighbors the active site. This interaction results in folding of the activation domain that allows access to the active site by CK2 substrates. Accordingly, the residues in the N-terminal segment and the activation domain that are involved in this interaction are highly conserved among CK2α from different species (Niefind et al. 1998). By comparison, the activation domain of human cyclin-dependent kinase CDK2 is folded away only after binding of cyclin A to the kinase (Jeffrey et al. 1995).

Consistent with the predominance of the tetrameric form of the kinase when isolated from cells, mixing of the α and β subunits in vitro results in the formation of a stable tetrameric holoenzyme by a self assembly mechanism. Initial studies indicated that the minimal fragment of human CK2β required for efficient formation of a dimer encompasses residues 20-165, thus suggesting that the interface between the β proteins is likely created by a three dimensional structure (Pinna and Meggio 1997; Boldyreff and Issinger 1997). This idea has since been corroborated by the crystal structure of human CK2β dimer; the region that spans the residues 105-146 is conserved and contains four invariable cysteines that fix a zinc atom (Chantalat et al. 1999). Extensive interactions between the zinc finger motifs form the interface between the two CK2β molecules. This structure is further stabilized by an extended C-terminal domain that contacts the other β subunit in the dimer (Niefind et al. 2001). The homodimerization of two regulatory subunits forms a stable structural framework in which simultaneous interactions of each β subunit with both catalytic subunits gives rise to a stable tetramer (Niefind et al. 2001).

Comparison of the activity of the free catalytic subunits to that of the native or reconstituted holoenzyme indicates that β subunit stimulates the kinase activity toward most substrates in vitro (Pinna and Meggio 1997). β also confers specificity to the catalytic subunits toward some substrates in vitro (Allende and Allende 1998). For instance the cAMP-responsive element binding protein and c-Jun are phosphorylated by CK2 catalytic subunits only in the presence of the regulatory subunit (Bodenbach et al. 1994). On the other hand, β largely abolishes phosphorylation of calmodulin and MDM-2 by the catalytic subunits (Bidwai et al. 1993; Guerra et al. 1999). While the structural basis for these observations are not clear, the extensive interactions

among the α and β subunits correlate with changes in the CD spectra which are indicative of conformational changes in the holoenzyme (Issinger et al. 1992; Jakobi and Traugh 1995). These conformational changes are believed to underlie the in vitro stimulation of the CK2α kinase activity by the bound β subunits (Niefind et al. 2001; Niefind et al. 1999). The proposed conformational changes in the CK2α are reminiscent of those induced in the catalytic domain of protein kinase A by its regulatory domain (Bossemeyer et al. 1993).

A highly acidic stretch of the β subunit that spans residues 55-64 is part of a prominent acidic ridge that is in close proximity to the enzyme catalytic pocket (Niefind et al. 2001). Although this structural feature makes no contact with the catalytic subunits, it can restrict access of negatively charged CK2 substrates to the active site by electrostatic repulsion (Niefind et al. 2001). Accordingly, substitution of the glutamic acid residues 60, 61, and 63 with alanine results in a 4 fold increase in activity of the CK2 holoenzyme in vitro (Leroy et al. 1999). The acidic stretch of CK2β also provides the binding site for polybasic compounds (Guerra et al. 1997). Binding of these compounds to this domain would relieve this hindrance by charge neutralization. This may explain the large increase in in vitro kinase activity of the CK2 holoenzyme, but not the catalytic subunit alone, by these compounds (Meggio et al. 1994; Guerra et al. 1997). The acidic domain of CK2β gives rise to a continuous and solvent exposed surface that should be available as a docking site for binding partners (Niefind et al. 2001). This raises the possibility that stretches of basic polypeptides may regulate the kinase activity by interacting with this CK2β domain (Chantalat et al. 1999). This domain is in fact implicated in binding to p53 and $p21^{WAF1}$ and, a carboxyl-terminal segment of p53 that contains the highly basic region 287-340 stimulates the kinase activity of the holoenzyme in vitro (Guerra et al. 1997; Gotz et al. 2000).

In contrast to the stimulatory effect of the polybasic molecules, some of the most potent in vitro inhibitors of CK2 are polyanionic compounds such as heparin (Pinna 1990). The structure of CK2α reveals that a highly conserved cluster of predominantely basic residues (K^{74}KKKIKREIL^{83}) is aligned toward the active site of the kinase (Niefind et al. 1998). Binding of heparin to the lysine-rich domain is thought to occlude the active site (Pinna 1990; Meggio et al. 1994). It is not known if

polyanionic compounds, such as heparin in the liver and 2,3 bisphosphoglycerate in red blood cells, play a role in regulation of the kinase in vivo (Meggio et al. 1990; Perich et al. 1990).

The minimum CK2 consensus site, largely determined by in vitro phosphorylation of the peptide substrates, is S/TxxE/D in which S/T denotes the phosphoacceptor serine or threonine. The acidic residue in the third downstream position is an important, but not absolute, determinant of the CK2 consensus (Allende and Allende 1998). It is thought that potential interactions of the acidic residues of the CK2 consensus site with the lysine-rich domain in the CK2α active site partly determine substrate recognition (Niefind et al. 1998). In addition to glutamic acid and aspartic acid, phosphoserine and phosphothreonine can also serve as acidic determinants. This allows not only for sequential phosphorylation of multiple Ser and Thr residues in a site (Meggio et al. 1988), it is also amenable to hierarchical phosphorylation of a site by CK2 and other Ser/Thr kinases (Krebs et al. 1988).

The absence of a "canonical" CK2 consensus site might explain its broad substrate specificity in vitro (Guerra et al. 1999). Among the in vitro substrates for CK2 are its regulatory subunits. The N-terminal serine 2 and serine 3 of the human CK2βare efficiently phosphorylated by the catalytic subunits of the enzyme (Bodenbach et al. 1994). The physiological significance of CK2 autophosphorylation is unknown since mutation of these sites has no effect on either the assembly or the activity of the CK2 holoenzyme (Bodenbach et al. 1994).

The ATP-binding site, characterized by key residues for ribose and triphosphate fixation, is a highly conserved feature in protein kinases (Knighton et al. 1991). A nearly invariant Ala that is in direct contact with the adenine base is among these residues. The equivalent position in maize CK2α is occupied by Ile66 that has a distinctively larger side chain (Val occupies the equivalent position in both human and yeast counterparts) that sterically hinders the binding of adenine (Niefind et al. 1998). This results in "non-stringent" binding of the co-substrate which, in turn, allows for efficient utilization of either ATP or GTP by CK2 (Niefind et al. 1999). Importantly, in vitro binding of CK2β to the catalytic subunit induces a subtle conformational change in an α-helix domain within the active site that is in direct contact with the co-substrate (Jakobi and Traugh 1995). This structural change is postulated to stabilize

the binding of the co-substrate and may partly explain the in vitro stimulation of CK2α catalytic activity by the β subunit .

The biological functions of CK2

In *S. cerevisiae*, deletion of either one of the genes encoding α and α' catalytic subunits results in no known phenotype, suggesting some degree of functional redundancy between the catalytic subunits (Bidwai et al. 1992). However, deletion of both genes is lethal, demonstrating that CK2 is essential for viability in yeast (Padmanabha et al. 1990). Cells with disruption of both catalytic genes are rescued by expression of the wild type, but not the active site mutant, *Drosophila* α subunit, indicating that the phosphotransferase activity of the enzyme is essential for its cellular functions (Glover et al. 1994).

A large number of CK2 in vitro substrates are involved in the regulation of gene expression, signal transduction, and cell cycle progression (reviewed in Glover 1998; Guerra et al. 1999; Guerra and Issinger 1999). Accordingly, CK2 has been implicated in these cellular processes. The outcome of these studies are discussed below.

CK2 and proliferation

A higher level of CK2 activity is generally found in proliferating cells, both normal and transformed (Heriche et al. 1997; Bailly et al. 2000). Furthermore, slight overexpression (>10% of the endogenous CK2α mRNA) of the catalytic subunit in the T cells of the transgenic mice is inducive to cellular transformation (Seldin and Leder 1995; Kelliher et al. 1996). These observations indicate a positive role for CK2 in cellular proliferation. Accordingly, the expression of CK2α and CK2α' is coordinately increased following serum stimulation of the quiescent human fibroblasts (Bosc et al. 1999). Conversely, antisense, antibody, or peptide substrate inhibition of CK2 in quiescent cells blocks mitogen-induced proliferation (Lorenz et al. 1994; Lorenz et al. 1999).

Yeast that harbor deletions of both catalytic subunits and only carry a temperature sensitive allele of CK2α' (cka1Δ cka2^{ts}) arrest in G1 and G2/M at the non-permissive temperature (Bidwai et al. 1992). A requirement for CK2 in G1

progression in yeast is in agreement with the reported increase in soluble CK2 activity in extracts prepared from G1 synchronized HeLa cells (Marshak and Russo 1994). Additionally, microinjection of the purified CK2α' into *Xenopus* oocyte potentiates mitosis promoting factor-induced maturation, thus implying a role for CK2 in mitotic progression (Belle et al. 1990; Mulner-Lorillon et al. 1990).

The G2/M arrested yeast CK2 mutants proceed into anaphase and then become inviable (Bidwai et al. 1992). This suggests that CK2 functions in execution of the events required for M phase progression downstream of cyclin-dependent kinase Cdc28, a key regulator of the cell cycle progression. The precise function of CK2 in mitotic progression is not clear (Meggio et al. 1995; Bosc et al. 1999). However a plausible target that can partly mediate this effect is topoisomerase II. Transient generation and rejoining of double strand DNA breaks by this enzyme is required for chromosome condensation and separation of intertwined chromosomal DNA during mitosis (Adachi et al., 1991). Similar to CK2 mutants, topoisomerase II mutants become inviable in M phase (Holm et al. 1985). In yeast, topoisomerase II is phosphorylated in M phase and this phosphorylation is abolished at the non-permissive temperature in a conditional CK2 mutant (Cardenas et al. 1992; Cardenas and Gasser 1993). Furthermore, dephosphorylation of topoisomerase II results in loss of activity in vitro, whereas its subsequent phosphorylation by CK2 restores enzymatic activity (Cardenas and Gasser 1993). CK2 is recovered in complex with topoisomerase II in both yeast and human, thereby suggesting that this regulatory mechanism may mediate the reported role of CK2 in eukaryotic M phase progression (Saijo et al. 1992; Bojanowski et al. 1993)

On the other hand, *S. cerevisiae* that harbor deletions of both catalytic subunits and carry only a conditional mutant of the CK2α (cka1^{ts} cka2Δ) exhibit no cell cycle arrest phenotype (Bidwai et al. 1994). Instead, these mutants exhibit defects in cell polarity. The cytoskeleton plays an essential role in maintaining the cell shape and polarity both in yeast and mammals (Faust et al. 1999). Immunostaining of rat brain indicates that CK2 is associated with microtubules and tubulin is a CK2 substrate in vitro and in vivo (Sarno et al. 1993; Faust et al. 1999).

The apparent phenotypical difference resulting from the inactivation of α and α' is surprising since cells expressing either of the catalytic subunits are

phenotypically indistinguishable (Bidwai et al. 1994). Differential function of CK2 catalytic subunits has also been indicated in mammalian cells where overexpression of kinase inactive CK2α', but not inactive CK2α, results in significant attenuation of proliferation in human osteosarcom cells (Vilk et al. 1999). These observations suggest a degree of functional specialization for α and α'. This is in agreement with the in vivo identification of isoform-specific interacting partners for the catalytic subunits (Heriche et al. 1997; Bosc et al. 2000). The divergence in amino acid sequence between α and α' (nearly 50% in yeast) provides a rationale for their functional specificity (Guerra and Issinger, 1999).

The regulatory subunits of CK2 greatly modulate the activity of the catalytic subunits, and in part determine the substrate specificity in vitro (Glover, 1998). However, *S. cerevisiae* with deletion of either or both CK2β and CK2β' genes are viable and, unlike the catalytic subunit mutants, only display a modest sensitivity to high salt (Bidwai et al. 1994). An obvious interpretation of this finding is that in *S. cerevisiae*, the catalytic subunits can be functionally autonomous in vivo. This is consistent with the observation that expression of *Drosophila* α subunit in yeast that harbor deletions of both catalytic subunits restores wild type phenotype despite its inability to interact with either of the yeast CK2 regulatory subunits (Padmanabha et al. 1990).

Unlike *S. cerevisiae*, deletion of the gene encoding the CK2β subunit of *S. pombe* results in markedly slower growth and abnormal rounded morphology (Roussou and Draetta 1994). The studies in higher eukaryotes, similar to the findings in *S. pombe*, also support a role for CK2β in proliferation. The level of CK2β in exponentially growing human fibroblasts is reported to be 2-3 folds higher than in quiescent cells (Pinna and Meggio 1997). Conversely, injection of a monoclonal antibody against CK2β significantly inhibits the resumption of cell cycle following mitogenic stimulation of the quiescent cells (Lorenz et al. 1999) or cells synchronized in G1 (Pepperkok et al. 1994). These data imply that CK2β participates in transmission of the mitogenic signals or cell cycle progression. In contrast to these reports, even a slight overexpression of CK2β prolongs the progression of fibroblasts and Chinese hamster ovary cells through G1 by reducing the expression of cyclin D1 (Li et al. 1999). It further results in a lower mitotic index by reducing the activity of

the mitosis promoting factor $p34^{cdc2}$ (Li et al. 1999). The likelihood of cell type-specific CK2β functions notwithstanding, these findings raise the possibility that in higher eukaryotes the optimal relative stoichiometry of the catalytic and the regulatory subunits are likely essential for CK2 functions. This is supported by the observation in which coordinated overexpression of the catalytic and regulatory subunits results in no significant change in proliferation in human osteosarcoma cells (Vilk et al. 1999; see below).

CK2 and signaling

Consistent with its localization to plasma membrane, cytoplasm, and nucleus, along with its in vitro phosphorylation of several signaling molecules, CK2 has been implicated in mediating signaling events. A compelling example of CK2 signaling function is provided by its regualtion of the mitogen activated protein kinase (MAPK) pathway. Protein phosphatase 2A (PP2A) downregulates MAPK pathway by dephosphorylating both MAPK and its upstream activator MEK1 (Sontag et al. 1993). CK2α, but not the β-containing holoenzyme, associates with and activates PP2A both in vivo and in vitro (Heriche et al. 1997; Lebrin et al. 1999). The in vivo association of CK2α with PP2A is mitogen-sensitive since treatment of quiescent human fibroblasts with growth factors disrupts their association. The functional significance of this association is demonstrated by a marked reduction in transforming activity of the constitutively active Ras^{val12} following overexpression of wild type CK2α, but not a mutated version that does not interact with PP2A (Heriche et al. 1997). The inhibition of MAPK signaling pathway by CK2α indicates that the monomeric form of CK2α can autonomously regulate a signaling event.

CK2 has also been implicated in signaling events at the plasma membrane. The transmembrane protein CD5 modulates antigen receptor-mediated activation of T cells and a subset of B cells. CK2 associates with CD5 via interaction of its β subunit with the cytoplasmic domain of CD5 (Raman and Kimberly 1998; Raman et al. 1998). Cross linking of CD5 leads to the activation of CD5-associated CK2 in thymocytes. CD5, in turn, is an efficient in vitro substrate of the activated CK2. As such, CK2 is both a target and a likely effector of the CD5-dependent signaling events.

Unlike many other kinases, CK2 remains stably associated with many of its substrates, and in fact some CK2 functions are mediated by its non-phosphorylating interactions (Johnson et al. 1996; Lorenz et al. 1999). Therefore, numerous studies have been aimed at identifying CK2 interacting partners in order to gain insight into its physiological functions. The employed biochemical analyses, and in particular yeast two-hybrid screens, have yielded a large and diverse array of CK2 interacting proteins (Yamaguchi et al. 1998; Gotz et al. 1999; Grein et al. 1999; Kusk et al. 1999). An alternative approach to understanding the in vivo functions of CK2 is to determine its physiological regulation. The fundamental logic behind this approach is that variation in CK2 activity that is concomitant with a cellular event may be causal to the initiation, or required for the execution of the event. The outcome of some these studies are discussed in the following section.

The Regulation of CK2

The catalytic subunits of CK2 either alone or within the tetrameric complex are constitutively active and, unlike many other kinases, CK2 does not respond to any known secondary messengers (Filhol et al. 1995). The pleiotropic functions of CK2 in the cell, however, suggest a requirement for its regulation. Therefore the in vivo regulation of CK2 has been the subject of extensive studies.

A potential regulatory mechanism is the variation in CK2 expression. Consistent with the role of CK2 in proliferation, the amount of CK2 mRNA and proteins is reported to be higher in tumors and transformed cells compared to normal cells (Robitzki et al. 1993). Furthermore, serum stimulation of quiescent fibroblasts increases the expression of CK2 subunits (Orlandini et al. 1998). Conversely, identification of SUN2, the regulatory subunit of the 20S proteosome, as a multicopy suppressor of a yeast CK2α temperature sensitive allele suggests that CK2 regulation may be mediated by specific proteolytic degradation (Glover et al. 1994). In proliferating human lymphocytes, the β subunit synthesized in excess of the α subunit is degraded (Luscher and Litchfield 1994). CK2β contains a destruction box similar to those found in cyclins that may mediate its degradation by ubiquitination-dependent proteolysis (Allende and Allende 1998). The degradation of the free β subunit may, in turn, regulate the activity of cellular CK2 by altering the relative stoichiometry of the

α and β subunits. Consistent with this idea, asymmetric distribution of the CK2 subunits in different tissues from mice has been reported (Xu et al. 1999); while CK2α is widely expressed in nearly all tissues in mice, CK2α' and CK2β are preferentially expressed in brain, testis, and mature spermatozoa. The physiological consequence of such asymmetric distribution is demonstrated by the presence of sperm anomaly in mice lacking CK2α' (Xu et al. 1999). The non-stoichiometric distribution of the RNA encoding the CK2 subunits is also demonstrated in various human adult tissues (Bosc et al. 2000).

The asymmetric distribution of the CK2 subunits is potentially significant since independent interactions of CK2α and CK2β with cellular proteins have been reported (Grein et al. 1999; Kusk et al. 1999; Leroy et al. 1999); yeast two hybrid screen of human cDNA libraries have yielded A-Raf as a CK2β–interacting partner (Boldyreff and Issinger 1997; Hagemann et al. 1997). A-Raf is one of three Raf kinases involved in MAPK signaling pathway that mediates fundamental processes such as cellular proliferation and differentiation (Daum et al. 1994). The biological significance of this interaction is suggested by the activation of Raf kinase following expression of murine CK2β in Sf9 cells (Hagemann et al. 1997). A similar screen of *Xenopus* cDNA has identified Mos as a CK2β interacting protein (Chen et al. 1997). Mos is a germ cell-specific serine/threonine kinase that is required for *Xenopus* oocyte maturation (Vande Woude et al. 1990). Active Mos stimuates MAPK signaling pathway by phosphorylating MAPK kinase (MKK). CK2β binds Mos and inhibits Mos-mediated activation of MKK activity in vitro (Chen et al. 1997). Moreover, microinjection of CK2β RNA into *Xenopus* oocyte inhibits MAPK-mediated meiotic maturation. The reason for apparently different roles of CK2β in MAPK activation in human somatic cells and its inactivation of this pathway in *Xenopus* germ cells is not clear. These observations, however, suggest that CK2β can function autonomously. This is supported by the findings that CK2β-mediated activation of A-Raf and its inhibition of Mos is abolished by co-expression of CK2α. Functional autonomy of CK2β is also suggested by studies in *S. pombe* in which overexpression of CK2β results in defective cytokinesis and impaired growth, whereas, CK2α overexpression confers no obvious phenotype (Roussou and Draetta 1994). The asymmetric expression of CK2β relative to the CK2 catalytic subunits in different tissues and in

human tumors might in fact reflect a cellular requirement for their autonomous functions (Stalter et al. 1994; Xu et al. 1999).

Despite the role of CK2 in cell cycle progression, some studies indicate that there are no significant variations in either the expression or the activity of CK2 throughout the cell cycle (Kikkawa et al. 1992; Bosc et al. 1999). Given the constitutive activity of CK2, it is postulated that the kinase activity might be regulated through its interaction with specific cell-cycle dependent inhibitors (Glover 1998). This mechanism is not without precedent since some cell cycle-dependent protein kinases are regulated by their interactions with protein inhibitors that are differentially expressed throughout the cell cycle (Ferguson et al. 1986; Belle et al. 1990). One such potential modulator of CK2 activity is $p21^{WAF1}$. p21 negatively regulates progression through G1 and G2/M by binding and inhibiting several cyclin-dependent kinases (Niculescu et al. 1998). It also binds the β subunit of CK2 both in vitro and in vivo and inhibits the enzyme's kinase activity in vitro (Gotz et al. 1996).

A variety of biochemical procedures including immunocytochemistry and cell fractionation have demonstrated that CK2 is present in nearly all cellular compartments (reviewed in Faust and Montenarh, 2000). In particular, the nuclear and cytoplasmic localization of the kinase is extensively studied. Both CK2α and CK2α' contain putative nuclear localization signals (Faust and Montenarh 2000). CK2β lacks a canonical nuclear localization signal, but may be targeted to the nucleus by binding to the shuttling nuclear import factor Nopp140 (Li et al. 1997). That the differential localization of CK2 might constitute a regulatory mechanism is supported by the finding that proliferating murine culture cells exhibit intensive nuclear staining with an anti-CK2α antibody, whereas in differentiated cells CK2α is predominantly cytoplasmic (Diaz-Nido et al. 1994). Furthermore, in prostate cancer cells CK2α translocates from the cytosol to the nucleus in response to androgen or certain growth stimuli (Guo et al. 1998; Guo et al. 1999). Conversely, androgen deprivation in rats decreases the abundance of nuclear CK2α in prostate cells (Tawfic et al. 1993; Tawfic et al. 1994). Collectively, these results suggest that the nuclear and cytoplasmic CK2 are functionally distinct. Consistent with this idea, microinjection of anti-CK2α or CK2α' antibodies or their substrate peptides results in significant inhibition of serum stimulation when injected into the nucleus but not the cytoplasm

of human fibroblasts (Lorenz et al. 1999). Importantly, similar inhibition of proliferation is observed following microinjection of pseudosubstrate peptides into the nucleus, implying that the effect of CK2 on proliferation is partly mediated by its non-phosphorylating interactions (Lorenz et al. 1999).

The diversity of the regulatory mechanisms discussed above point to the complexity of the CK2 regulation. Importantly, these mechanisms are not mutually exclusive and raise the possibility that the in vivo regulation of CK2 may be combinatorial.

CK2 and transcription

The regulation of transcription throughout the cell cycle and in response to a variety of signaling pathways has been well documented (reviewed in Huet et al. 1996; Warner 1999; White et al. 1995). The usual end point of these pathways is the activation or inactivation of transcription factors which eventually results in differential gene expression. The observation that some changes in gene expression can occur in the absence of protein synthesis indicates that they can be mediated by post translational modifications of transcription factors.

Among such protein modifications, phosphorylation is arguably the most extensively employed (reviewed in Hunter and Plowman, 1997). Phosphorylation has a number of features that make it ideally suited for regulating the activity of the transcription factors. It can be exceedingly rapid; changes in phosphorylation of some transcription factors in response to external stimuli are essentially complete within minutes (Buratowski et al. 1988). Phosphorylation is readily reversible by phosphatases that exhibit kinetics similar to that of the kinases (Wang et al. 1995). Furthermore, since a transcription factor can be targeted by several kinases, phosphorylation can integrate information from multiple signaling pathways (Hunter and Plowman 1997).

Consistent with the localization of CK2 to both the nucleoplasm and the nucleolus and the large number of transcription factors among its in vitro substrates, CK2 has been implicated in the regulation of transcription by all three RNA polymerases.

CK2 and pol I transcription

Transcription of the ribosomal RNA genes by RNA polymerase I correlates with cell growth and is greatly inhibited in confluent cells (Belenguer et al. 1989). Immunocytochemical analysis shows intensive staining of CK2 in the nucleoli, the site of pol I transcription, of dividing bovine endothelial cells. In confluent cells, however, CK2 is absent from the nucleoli. Importantly, addition of exogenous CK2 restores the transcription in nuclei isolated from the confluent cells, thus suggesting a role for CK2 in pol I transcription (Belenguer et al. 1989).

RNA polymerase I exists as a preassembled holoenzyme that contains associated transcriptional activators and repressors in addition to its core subunits (Kim et al. 1994; Li et al. 1996). In agreement with a role for CK2 in pol I transcription, CK2 cofractionates with RNA polymerase I holoenzyme through multiple chromatography steps (Albert et al. 1999). CK2α also coimmunoprecipitates with, and in vitro phosphorylates, the largest subunit of the mammalian RNA polymerase I (Hannan et al. 1998). However, the functional significance of CK2 association with pol I remains unclear. CK2 is also implicated in the regulation of murine Upstream Binding Factor (UBF), a homodimer that binds to pol I enhancer and activates transcription by facilitating the binding of TBP-containing factor to the core promoter (Voit et al. 1992). UBF is a phosphoprotein in vivo and this phosphorylation is required for its transcriptional activity in vitro. Phosphopeptide mapping suggests that CK2 may contribute to in vivo UBF phosphorylation (Voit et al. 1995). The present study further supports a role for CK2 in pol I transcription by demonstrating that rRNA synthesis is impaired in a *S. cerevisiae* CK2 mutant in vivo (see Chapter 2).

CK2 and pol II transcription

Addition of CK2 substrate peptide to the HeLa extract inhibits elongation by the RNA polymerase II and the extent of the inhibition is proportional to the efficiency of the peptide as a CK2 substrate in vitro (Angiolillo et al. 1993). This implies a requirement for CK2 phosphorylation in pol II transcription. Immunolocalization of CK2α indicates that it colocalizes with the transcribing RNA polymerase II enzyme on the active gene loci (Zandomeni et al. 1986). Furthermore,

treatment of human colon cells with the ATP analog DRB, a potent inhibitor of CK2, inhibits polmerase II elongation (Egyhazi et al. 1982). Following DRB treatment, there is a time dependent clearance of CK2α and RNA polymerase II from the transcribing loci (Egyhazi et al. 1982). Significantly, purified CK2 partially restores pol II transcription when added to an extract from DRB-treated HeLa cells (Zandomeni et al. 1986). More recent studies have revealed that DRB is also an inhibitor of cyclin-dependent kinase CDK7 (Yankulov et al. 1995). Treatment of HeLa cells with DRB inhibits the phosphorylation of RNA polymerase II by its associated CDK7, thus effectively blocking promoter clearance by the polymerase. It may be that the transition from initiation to elongation requires pol II phosphorylation by CDK7, whereas phosphorylation of pol II during the subsequent elongation steps is CK2-mediated.

In addition to its function in basal pol II transcription, CK2 has been implicated in regulating the function of a variety of pol II transcriptional activators (Pinna and Meggio 1997; Yamaguchi et al. 1998). CK2α interacts with the basic leucine zipper (bZIP) domain of several transcriptional activators in vitro. This highly conserved domain mediates the DNA binding of some well-characterized transcriptional activators such as cyclic AMP-response element binding protein (CREB) and c-Jun. In quiescent human fibroblasts CK2 phosphorylates c-Jun and abolishes its DNA binding (Lin et al. 1992). Nuclear microinjection of a CK2 substrate peptide greatly stimulates the in vivo transcriptional activity of c-Jun. Similar phosphorylation of several other transcriptional activators by CK2 and their concomitant loss of DNA binding in vitro have been reported (Berberich and Cole 1992; Upton et al. 1995; Johnson et al. 1996; Gotz et al. 1999; Solow et al. 1999). However, it is not clear whether these observations reflect the in vivo regulatory mechanisms, since loss of DNA-binding by these factors may be due to their aberrant phosphorylation by CK2 in vitro.

CK2 and pol III transcription

Studies in our lab have provided the first demonstration of a requirement for CK2 in efficient pol III transcription in *S. cerevisiae* (Hockman and Schultz 1996). Transcription of tRNA and 5S rRNA genes is impaired in a CKa2^{ts} mutant both in

vivo and in vitro. The absence of an accumulation in intermediate RNA forms in the CK2 mutant argues against a role for CK2 in elongation or processing of pol III transcribed RNA. Instead, the marked reduction in the synthesis of primary transcripts in the mutant indicates a defect in pol III initiation. Importantly, addition of purified CK2 restores pol III transcription in extracts prepared from the CK2 mutant, thus suggesting a direct involvement for CK2 in pol III transcription (Hockman and Schultz 1996).

The work presented here confirms and extends these findings. The present study will demonstrate that in *S. cerevisiae*:

- CK2 stably associates with the TBP subunit of TFIIIB,
- Phosphorylation of TFIIIB by CK2 is required for efficient initiation of pol III transcription,
- pol III transcription is repressed in response to genotoxic stress and this repression is largely governed by the CK2-mediated regulation of TFIIIB,
- Similar to pol III, pol I transcription is regulated by CK2 in vivo, both during normal growth and in response to DNA damage. The CK2-mediated coupling of pol I and pol III transcription provides a plausible mechanism for coordinated regulation of cellular protein synthesis.

Figure 1-1. Crystal structure of TATA-bound yeast TBP. The TATA-containing sequence TATAAAAG (red) from the adenovirus major late promoter binds to the concave surface of TBP (blue) by bending towards the major groove. This produces a underwound minor groove which forms a primarily hydrophobic interface with the entire undersurface of TBP saddle. DNA photoaffinity labeling experiments suggest that the overall architecture of TATA-bound TBP is maintained in DNA-bound TFIIIB. Adapted from Kim et al. 1993.

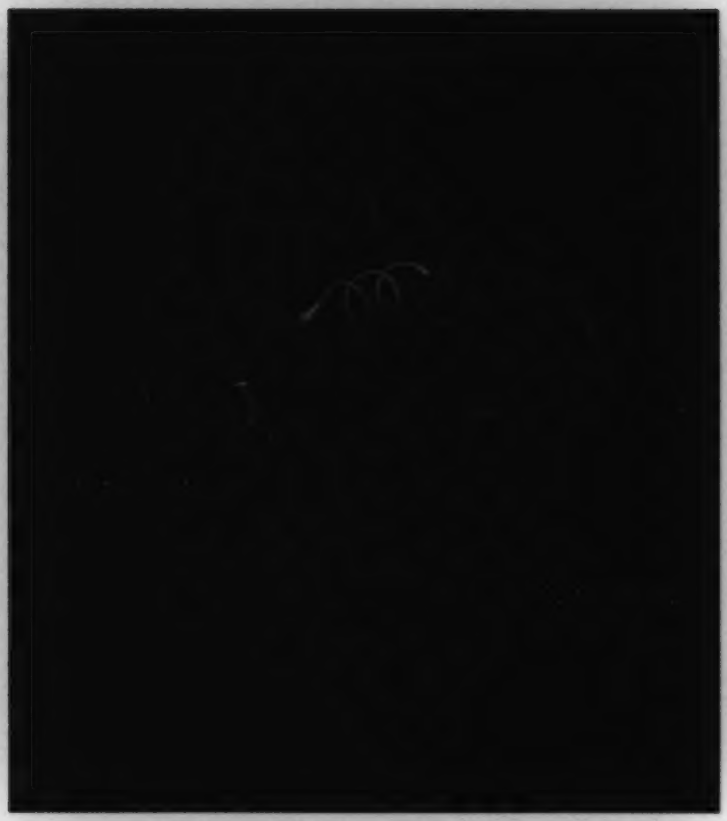


Figure 1-2. Crystal structure of Zea mays CK2α. The position of the active site is marked by the bound ATP molecule. The interaction between the N-terminus of the kinase and the activation domain (red), which neighbors the catalytic loop (purple), provides a structural basis for the constitutive activity of the kinase. The lysine rich domain $K^{74}KKKIKREIL^{83}$ (yellow) is aligned towards the active site of the kinase. Interactions of this domain with the acidic residues of the CK2 consensus site may partly mediate substrate recognition. Adapted from Niefind et al. 1998.

Chapter 2

Regulation of pol III transcription by CK2

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Introduction

The strict regulation of pol III transcription has been documented in diverse eukaryotic organisms (reviewed in White et al. 1995; Kassavetis et al. 1999). In higher eukaryotes, nuclear transcription is repressed during mitosis in vivo (Gottesfeld et al. 1994; White et al. 1995). All RNA transcription stops by midprophase and does not resume until late in telophase. This global repression of transcription is thought to be required for chromosomal condensation and separation of the replicated genome to occur without interference from the transcriptional apparatus (White et al. 1995). Mitotic repression of pol III is not merely a result of exclusion of transcription factors from DNA by nucleosomes. Rather, downregulation of pol III occurs via an active repression mechanism. Thus addition of recombinant cyclin B to transcriptionally active *Xenopus* interphase extract results in significant repression of transcription (Hartl et al. 1993). Furthermore, fractionated TFIIIB is inactivated by incubation with affinity purified mitotic kinase $p34^{cdc2}$ and cyclin B complex (Gottesfeld et al. 1994; Wolf et al. 1994). This reaction is inhibited by kinase inhibitors and requires a hydrolyzable nucleotide triphosphate (Wolf et al. 1994). Conversely, addition of TFIIIB restores pol III transcription in mitotic extracts (Gottesfeld et al. 1994). These results demonstrate that mitotic repression of pol III transcription is mediated by the cyclin-dependent kinase $p34^{cdc2}$ phosphorylation of TFIIIB.

Studies in HeLa cells show that pol III transcription is also repressed in early G1 cells in vivo (White et al. 1995). Transcription gradually increases as cells pass through the restriction point in G1 and reaches maximal level during S and G2 phase. This observation suggests that only after commitment to continue proliferation does pol III transcription increase to a level that is sufficient for sustaining growth. A requirement for induction of pol III transcription in G1 progression is in agreement

with the finding that an inactivating mutation of the p53 subunit of yeast RNA polymerase III enzyme results in predominantly late G1 arrest (Mann et al. 1992). Furthermore, addition of TFIIIB to extract from HeLa cells synchronized in G1 restores transcription (White et al. 1995). Collectively, these results indicate that cell cycle-dependent RNA polymerase III transcription is mediated by specific regulation of the core transcription factor TFIIIB.

The regulation of pol III transcription by the tumor suppressors retinoblastoma protein (Rb) and p53 clearly illustrates its role in normal growth. Rb restricts cellular proliferation and is frequently mutated in transformed cells (White et al. 1996). It binds TFIIIB and inactivates pol III transcription in quiescent fibroblasts, likely a reflection of diminished requirement for protein synthesis (Larminie et al. 1998; Scott et al. 2001; White et al. 1996). Upon mitogenic stimulation, Rb is phosphorylated by Ras-activated cyclin-dependent kinases. This results in dissociation of TFIIIB from the phosphorylated Rb and subsequent induction of pol III transcription (White et al. 1996). Various deletions within the C-terminal domain of Rb, along with several of its naturally occurring mutations that prevent its function as a tumor suppressor, also abolish its ability to repress pol III transcription (White et al. 1996). Accordingly, viral oncoproteins SV40 large T antigen and adenovirus E1A that bind and inactivate Rb, also result in induction of pol III transcription (White et al. 1996). Similar to Rb, the tumor suppressor p53 represses pol III transcription in vitro and many tumors containing inactivating mutations in p53 exhibit elevated levels of pol III transcription (Cairns and White 1998). These findings suggest that the repression of pol III transcription partly mediates growth arrest by tumor suppressors. It further implies that aberrant pol III transcription, likely due to a requirement for increased protein synthesis, may be among the hallmarks of cellular transformation.

In addition to its regulation according to growth rate and throughout cell cycle, modulations of pol III transcription by kinase-mediated signaling in response to external cues have been demonstrated; treatment of *S. cerevisiae* with the macrolide antibiotic rapamycin represses pol III transcription by interfering with the TOR (Target Of Rapamycin) signaling pathway (Zaragoza et al. 1998). Protein kinase C has also been implicated in induction of pol III gene expression in response to treatment of *Drosophila* Schneider cells with tumor promoting phorbol ester TPA

(Garber et al. 1991; Garber et al. 1994). Earlier studies in our lab have demonstrated a requirement for protein kinase CK2 in efficient pol III transcription in *S. cerevisiae* (Hockman and Schultz 1996). The studies presented in this chapter will 1) confirm and extend these results, 2) determine the target of CK2 among the pol III transcription machinery, and 3) provide a mechanistic basis for CK2 requirement in pol III transcription.

Results

CK2 is required for efficient pol III transcription in vivo

A requirement for CK2 in pol III transcription was examined in the isogenic strains CKA2 and cka2^{ts}. In both strains the chromosomal *CKA1* and *CKA2* genes, encoding α and α' subunits, respectively, are disrupted. Strain CKA2 carries a wild type copy of CK2α' on a centromeric plasmid, whereas cka2^{ts} carries a temperature sensitive version (Hanna et al. 1995). Since the CK2 catalytic subunits are functionally redundant in cellular growth, these strains are viable at the permissive temperature (25°C). While CKA2 cells maintain normal growth after shifting to the restrictive temperature (37°C), cka2^{ts} cells rapidly cease growth and become arrested in G1 and G2/M (Hanna et al. 1995). The heat shock-induced cell cycle arrest in cka2^{ts} cells is associated with a drastic loss of viability; heat shocking these cells for two hours results in greater than 50% cell death.

Although viable at the permissive temperature, cka2^{ts} cells, nevertheless, exhibit phenotypes distinct from CKA2 cells. These include elongated cellular morphology, increased flocculation, and reduced rate of growth (A.G. unpublished results; Hanna et al. 1995). Importantly, extracts prepared from cka2^{ts} cells grown at 25°C contain significantly reduced levels of CK2 kinase activity (Hockman and Schultz 1996). These observations suggest that CK2 is functionally compromised in cka2^{ts} cells even when grown at the permissive temperature. Therefore, throughout this study, the experiments in cka2^{ts} strain are conducted at the permissive temperature. This approach circumvents the terminal phenotype of these cells at the restrictive temperature that could potentially undermine data interpretation.

To investigate a requirement for CK2 in pol III transcription in vivo, tRNA synthesis was monitored in CKA2 and cka2^{ts} cells using an S1 nuclease protection assay (Hockman and Schultz 1996). Since tRNA introns are turned over in the cell with a half -life of less than 3 minutes, their abundance is an accurate indication of the in vivo level of transcription (Cormack and Struhl 1992). Total RNA was prepared from cells cultured at the permissive temperature, hybridized to the tRNA^{Leu} probe, and then digested with S1 nuclease (Fig. 2-1, A, upper panel). Equivalent RNA recovery and loading was also monitored by ethidium bromide staining of 25S and 18S rRNAs (Fig. 2-1, A, lower panel). The level of intron-containing tRNA is markedly reduced in cka2^{ts} cells. Impaired synthesis of pol III transcribed tRNAs and 5S rRNA is also indicated by ^{3}H-uracil metabolic labeling of CKA2 and cka2^{ts} cells at the permissive temperature (Fig. 2-1, B). Thus two independent assays demonstrate a requirement for CK2 in pol III transcription in vivo. The transcription profile of cka2^{ts} cells in panel B also reveals a defect in synthesis of pol I-transcribed 5.8S, 18S, and 25S. The coordinated regulation of pol I and pol III transcription by CK2 are discussed in detail in Chapter 5.

Impaired pol III transcription in CK2-deficient extract

Preparation of an extract from *S. cerevisiae* that supports pol III transcription in vitro has been reported (Schultz et al. 1992). The in vitro pol III transcription in these extracts provides an accurate reflection of the in vivo events and has been successfully used in characterization of pol III transcription in vitro (Schultz et al. 1992; Hockman and Schultz 1997). To further study the role of CK2 in transcription, extracts were prepared from CKA2 and cka2^{ts} cells grown at the permissive temperature. These extracts were initially assayed for bulk CK2 activity using a CK2 peptide substrate. The measured activity is largely dependent on exogenous CK2 peptide substrate, efficiently utilizes GTP as the phosphate donor, and is highly sensitive to heparin (Fig. 2-2, A). These properties indicate that the phosphorylating activity is largely due to CK2. Consistent with a previous report (Hockman and Schultz 1996), the CK2 activity is greatly diminished in cka2^{ts} cells grown at the permissive temperature (Fig. 2-2, A).

Similar to in vivo pol III transcription profile in cka2^{ts} cells, transcription of 5S rRNA is defective in cka2^{ts} whole cell and nuclear extracts (Fig. 2-2 panels B and C, respectively). The difference in pol III transcription between CKA2 and cka2^{ts} extracts persists over a range of protein concentrations, thus ruling out the possibility that the protein amount required for optimal transcription differs between the extracts. Furthermore, pol III transcription in cka2^{ts} extract, similar to its in vivo transcription (Fig. 2-1, B), is specifically defective at the level of primary 5S rRNA transcription. This suggests that impaired pol III initiation likely underlies the transcriptional defect in cka2^{ts} cells.

Impaired transcription in CK2- deficient extract is preserved in a pol III enriched fraction

The pol III transcription machinery can be partially purified by DEAE chromatography (Riggs and Nomura, 1990). This procedure yields a 300 mM KCl cut from DEAE, referred to as the D300 fraction, that efficiently transcribes 5S rRNA and tRNA templates (Fig. 2-3, panels A, C). An important feature of D300 fraction is its considerably lower content of chromosomal DNA compared to whole cell extract (personal observation). The contaminating DNA may non-specifically sequester transcription factors that have intrinsic DNA binding properties. In order to minimize the possible squelching effect of chromosomal DNA in the transcription assay, D300 fractions were prepared and assayed in parallel from CK2 and cka2^{ts} cells. The recovery of basal pol III factors, as preliminarily judged by TBP immunoblotting, is similar between the corresponding fractions from CK2 and cka2^{ts} (Fig. 2-3, B). The defect in CK2 kinase activity in whole cell extracts from these cells is also preserved in D300 fractions (panel E). When assayed for pol III transcription, D300 fractions maintain, and indeed accentuate, the difference between the whole cell extracts (Fig. 2-3, A). Furthermore, similar to whole cell extracts, the defect in pol III transcription in cka2^{ts} D300 fraction is displayed at the level of primary transcripts, further indicating a specific defect in pol III initiation (Fig. 2-3, C).

The impaired pol III transcription in CK2-deficient extract may be to due to reduced activity of pol III factor(s). Alternatively, pol III transcription may be actively repressed by a transcriptional repressor normally silenced by CK2. A mixing

experiment was performed to address this issue. Titering increasing amounts of cka2^{ts} extract (10 and 20 μg) into a fixed amount of CK2 extract (10 μg) does not result in repression of transcription in CKA2 extract (Fig 2-3, D). This result argues against the presence of a dominant transcriptional repressor in CK2-deficient extract. Instead, it supports the hypothesis that the transcriptional defect in CK2-deficient extracts is due to impaired activity of pol III factor(s).

Transcription in CK2-deficient extract is rescued by TFIIIB

In order to identify the CK2-responsive component of the pol III transcription machinery, cka2^{ts} extracts were complemented with active transcription factors fractionated from a wild type extract. This biochemical approach is feasible since the defect in pol III transcription in cka2^{ts} extract is due to impaired transcription apparatus and therefore, likely to be restored by addition of the functionally intact factor(s).

Initially, two complementing fractions from wild type cells, one enriched in polymerase III/TFIIIC (fraction P/C) and another enriched in TFIIIB were prepared (Fig. 2-4, A; fractionation scheme is depicted in Fig. 6-1, Chapter 6). Both wild type and cka2^{ts} extracts were responsive to TFIIIB, although the magnitude of the response differed significantly between the extracts. TFIIIB slightly stimulated transcription in wild type extract (Fig. 2-4, B, lanes 1-4). In contrast, TFIIIB fully restores transcription in cka2^{ts} extract (Fig. 2-4, B, lanes 5-8). This result suggests that the defect in cka2^{ts} extract results primarily from inactivation of TFIIIB. This conclusion is supported by complementation experiments using the P/C fraction. The P/C fraction only marginally stimulated cka2^{ts} extract (Fig. 2-4, C, lanes 5-8), thus suggesting that neither RNA polymerase III nor TFIIIC is significantly affected by the loss of CK2 activity.

In view of these results, it is expected that a cka2^{ts}-D300 fraction that is severely impaired compared to the wild type fraction would be highly responsive to the addition of TFIIIB. Indeed, TFIIIB greatly stimulates transcription in cka2^{ts}-D300 (Fig. 2-5, A). To confirm that this stimulatory effect corresponds to TFIIIB activity, cka2^{ts}-D300 was supplemented with aliquots of fractions from the hydroxylapatite column used for TFIIIB purification (Fig. 6-1, Chapter 6). The activity that stimulates

cka2^{ts}-D300 occurs in fractions 34-38, with the peak in fraction 36 (Fig. 2-5, B). This profile corresponds to TFIIIB activity that complements the P/C fraction (Fig. 2-4, A) in an in vitro pol III reconstitution assay. Moreover, the results of adding P/C to whole cell extract of cka2^{ts} cells suggest that pol III and TFIIIC are largely unaffected by CK2 (Fig. 2-4, C). This interpretation is supported by the results obtained when the P/C fraction is titrated into the respective D300 fractions: P/C produced a slight inhibition of transcription in CKA2-D300 (Fig. 2-5, C, lanes 1-3) and marginally stimulated cka2^{ts}-D300 (lanes 4-6).

The transcriptional defect in CK2-deficient extract is not due to inactivation of TFIIIC or RNA polymerase III

The add back experiments above suggest that in CK2-deficient extracts both TFIIIC and pol III remain transcriptionally active. To further examine this possibility, TFIIIC and pol III in CK2 and cka2^{ts} extracts were directly assayed. In a gel mobility shift assay, TFIIIC efficiently binds to an end-labeled 319 bp DNA probe that encompasses the entire coding region plus 50 bp upstream region of a yeast tRNA^{Tyr} gene (Fig 2-6, lanes1-3; Kassavetis et al. 1990). The formation of the TFIIIC-DNA complex is inhibited by the wild type, but not the binding site mutant competitor (panel A, lanes 4-9). Using this assay no difference in DNA binding by TFIIIC in CKA2 and cka2^{ts} extracts was detected (panel A, lanes 10 and 11). Furthermore, bulk RNA polymerase III activity, as determined by the rate of transcriptional elongation, was virtually identical in CKA2 and cka2^{ts} whole cell extracts (Fig. 2-6, B). Collectively these assays indicate that in cka2^{ts} extracts both TFIIIC and pol III maintain their transcriptional activities.

Transcriptional activity of TFIIIB is specifically impaired in a cka2^{ts} extract

Taken together the results in Figures 2-4 through 2-6 indicate that inactivation of CK2 results in impaired activity of the TFIIIB component of the pol III transcription machinery. To directly verify this finding, TFIIIB was fractionated in parallel from CKA2 and cka2^{ts} cells. These fractions show similar protein profiles as judged by silver stained materials and their TBP and Brf1 contents (Fig. 2-7, A and B). TFIIIB from cka2ts cells is, however, impaired in reconstitution of pol III

transcription in combination with wild type RNA polymerase and TFIIIC (P/C) fraction (Fig. 2-7, C). This result provides further evidence in support of a requirement for CK2 in efficient activity of the basal pol III transcription factor TFIIIB.

Efficient phosphorylation of the TBP subunit of TFIIIB by CK2 in vitro

The cellular functions of CK2 are largely mediated by its phosphorylating interactions (see Chapter 1). Accordingly, the defect in pol III transcription occurs in response to the loss of CK2 catalytic activity. This raises the possibility that efficient TFIIIB function requires its phosphorylation by CK2. To examine this idea, the Cibacron Blue TFIIIB fraction was incubated with CK2 in the presence of γ^{32}P-GTP as the phosphate donor. As an internal control, fractionated TFIIIC was similarly subjected to CK2 phosphorylation. Since the transcriptional activity of TFIIIC is not affected by CK2, it is not expected to be a CK2 substrate. The reaction products were initially analyzed by TBP immunoblotting (Fig. 2-8, B). Following immunodetection the membrane was stripped of antibody and ^{32}P-labeled proteins were detected by autoradiography (Fig. 2-8, A).

When incubated alone, yeast CK2 autophosphorylates its β and β' subunits (Fig. 2-8, A, lane 1; Bidwai et al. 1994). TFIIIC does not contain significant GTP-dependent protein kinase activity on its own, nor is it an apparent CK2 substrate in vitro (panel A, lanes 2, 3). The absence of TFIIIC phosphorylation, despite the fact that several of its subunits contain multiple CK2 sites (Conesa et al. 1993), argues for a degree of CK2 substrate specificity in vitro. On the other hand, when TFIIIB is used as a substrate for CK2, a single band in addition to the regulatory subunits of CK2 is efficiently labeled (panel A, lane 5). This band, similar to yeast TBP, migrates with an apparent molecular weight of 27 kDa and is present only in the TBP-containing TFIIIB (panel B). Additionally, recombinant yeast TBP, expressed in *E. coli* and purified to near homogeneity, is a CK2 substrate and precisely comigrates with the phosphorylated TFIIIB component (Fig. 2-8, C). These preliminary observations raise the possibility that phosphorylation of TBP by CK2 is required for efficient transcriptional activity of TFIIIB.

TBP and a limiting amount of CK2 restore transcription in CK2-deficient extracts

To examine the possibility of a direct role for CK2 in transcription, increasing amounts of purified yeast CK2 holoenzyme were titrated into a cka2^{ts} nuclear extract in order to restore pol III transcription (Fig. 2-9, A). Addition of CK2 results in partial stimulation of transcription. The reason for inability of CK2 to fully restore transcription in the defective extract is not fully understood. A plausible explanation for this effect is provided by the rapid self-aggregation, and a concomitant loss of activity, of CK2 under low salt conditions similar to the ionic concentration used in the transcription assay (Miyata and Yahara 1992). In vitro oligomerization of CK2 is dose-dependent, which may explain the observed modest effect of CK2 in transcription even when added in exceedingly higher amounts.

The functional significance of in vitro TBP phosphorylation by CK2 was next examined by supplementing a CK2-deficient nuclear transcription extract with recombinant yeast TBP and purified yeast CK2, either alone or in combination (Fig. 2-9, B). Addition of TBP alone significantly stimulates transcription in cka2^{ts} extract (panel B, lanes 1-3). Adding larger amounts of TBP inhibits transcription (lane 4), likely by non-specific occlusion of the template. The observed stimulatory effect of TBP suggests that a TBP-dependent step in transcription is impaired as a consequence of CK2 depletion. The effect of adding TBP and CK2 together supports this idea. The optimal amount of TBP required to maximally stimulate transcription in cka2^{ts} extract is significantly lowered when TBP is added along with a limiting amount of CK2 (compare lanes 2 and 6, panel B). Importantly, under these conditions stimulation by CK2 and TBP when added together is significantly greater than the total stimulation by each component added individually (lanes 2, 5 and 6). A similar result is obtained when TBP and CK2 are added to the D300 fraction of cka2^{ts} (Fig. 2-9, C). These experiments demonstrate that the repression of TFIIIB resulting from inactivation of CK2 can be overcome by bacterially expressed TBP in the presence of a limiting amount of CK2. They further indicate functional cooperativity between TBP and CK2. A plausible interpretation of these results is the presence of a direct biochemical interaction between TBP and CK2.

TFIIIB must be phosphorylated to restore transcription in cka2^{ts} extract

The efficient phosphorylation of TBP by CK2 in vitro, along with their functional synergy in restoring transcription, raises the possibility that phosphorylation of TBP by CK2 is required for efficient TFIIIB function. This idea is consistent with the impaired transcriptional activity of TFIIIB from cka2^{ts} cells (Fig. 2-7, C). An obvious implication of this idea is that dephosphorylated TFIIIB should be transcriptionally impaired. This was examined by assaying the ability of dephosphorylated wild type TFIIIB to restore transcription in cka2^{ts} extract.

TFIIIB was treated with calf intestine alkaline phosphatase (CIP) in the presence or absence of the phosphatase inhibitor, sodium vanadate, then added to cka2^{ts} nuclear extract (Fig. 2-10). TFIIIB retained its capacity to stimulate cka2^{ts} extract when incubated with phosphatase in the presence of sodium vanadate (lanes 1,3,6,7). In contrast, TFIIIB treated in turn with phosphatase and then sodium vanadate was unable to stimulate cka2^{ts} extract (lanes 4,5,6,7). This experiment demonstrates that phosphorylation of TFIIIB is required for its function in pol III transcription.

Point mutation of a putative CK2 phosphorylation site in TBP results in impaired pol III transcription in vivo

Yeast TBP contains four CK2 consensus sites (Fig. 2-11, A). Since the amino terminal 60 residues are dispensable for in vivo functions of TBP, potential CK2 sites in this region (Ser 42 and Ser 53) are unlikely to play a significant role in pol III tarnscription. The other sites (Ser 128 and Ser 183) are located within the essential domain of TBP and are highly conserved. Modelling studies indicate that Ser 183, which is buried in the crystal structure of TBP, is unlikely to be solvent exposed (Chasman et al. 1993). This residue is therefore unlikely to be available for phosphphorylation by CK2. On the other hand, Ser 128 is solvent exposed and in fact located within a small domain of TBP already implicated in pol III transcription in both yeast and human cells (Cormack and Struhl, 1993, Bryant et al. 1996). This residue was substituted with either alanine (S128A) or aspartic acid (S128D) in order to study the possible effect of TBP phosphorylation by CK2 in pol III transcription. Substitution with alanine abolishes the putative CK2 phosphorylation site, whereas

aspartic acid provides a charge mimic for constitutively phosphorylated serine. The respective S128A and S128 D mutants grow at a slightly slower rate (as judged by the size of their colonies) at the permissive temperature (Fig. 2-11, B, upper panel) and this defect becomes more pronounced after shifting the cells to the restrictive temperature (B, lower panel). Although the TBP mutants display markedly slower growth than the wild type at 37°C, they however maintain viability even after extended heat shock treatments (personal observation).

Whole cell extracts and total RNA were prepared from the wild type and the TBP mutants after 90 minutes of heat shock. The similar TBP content among these extracts indicates that the point mutations do not affect TBP expression or stability (Fig 2-12, A). The apparent migration of S128D TBP in SDS-PAGE notably differs from that of the wild type. Similar anomalies in migration of human TBP following point mutation of some its residues have been reported (Bryant et al. 1996).

The S1 nuclease assay indicates decreased levels of newly transcribed tRNA in both TBP mutants (Fig. 2-12, B). The observed transcriptional defect in cells with the phosphomimetic TBP S128D substitution may be an indication of a fundamentally compromised TBP molecule, likely due to mutation-induced conformational changes as indicated by the aberrant migration of the S128D TBP. An alternative explanation for the reduced pol III transcription in mutant S128D is provided by the observation that an acidic residue need not functionally replace a phosphorylated serine in vivo (Dever et al. 1992). Therefore, the S128D substitution may not functionally represent phosphorylated Ser 128 of TBP. The diminished pol III activity in S128A mutant is, nevertheless, in agreement with the hypothesis that efficient TFIIIB function requires phosphorylation of its TBP subunit.

CK2 controls promoter recruitment of TFIIIB

The transcriptional profile of cka2^{ts} cells indicates a specific defect in pol III initiation (Figures 2-1, 2-2). The early steps in pol III transcription initiation are promoter binding of TFIIIC and subsequent recruitment of TFIIIB by DNA-bound TFIIIC (Kassavetis et al. 1993). Since wild type and cka2^{ts} TFIIIC have similar DNA binding properties (Fig. 2-6), impaired recruitment of TFIIIB likely underlies the transcription defect in CK2-deficient extract. Accordingly, the promoter recruitment of TFIIIB was examined by a gel mobility shift assay (Fig. 2-13, Kassavetis et al.

1990). Following the recruitment of TFIIIB to the promoter, heparin was used to strip TFIIIC from DNA (lanes 2, 3). Due to its stable DNA binding, TFIIIB remains associated with the promoter after heparin treatment (lane 5). The formation of IIIB-DNA complex is confirmed by its apparent supershifting in the presence of affinity purified anti-TBP antibody (lanes 6-11).

In order to examine their ability to form initiation complexes, TFIIIB from CKA2 and cka2^{ts} cells were assayed by gel mobility shift. TFIIIB from wild type cells readily formed a heparin-resistant IIIB-DNA complex (Fig. 2-14, A, lanes 1-4). On the other hand, TFIIIB purified from cka2^{ts} cells was significantly defective in generating the IIIB-DNA complex (lanes 5-8). The observed defect in TFIIIB promoter recruitment provides a mechanistic basis for its impaired transcriptional activity in vitro.

Dephosphorylation of TFIIIB greatly diminishes its transcriptional activity in vitro (Fig. 2-10). Consistent with this observation, TFIIIB treated with phosphatase followed by phosphatase inhibitor (Fig. 2-14, B, lanes 2-4) has a substantially reduced complex forming ability than untreated TFIIIB (lane 1), or TFIIIB to which the phosphatase and the inhibitor have been added following a brief coincubation (lanes 5-7). Collectively, these experiments demonstrate that CK2-phosphorylation of TFIIIB is required for its efficient recruitment to promoters and full transcriptional activity in vitro.

Discussion

The results presented in this Chapter confirm a previous report (Hockman and Schultz 1996) that the enzymatic activity of the ubiquitous protein kinase CK2 is required for high-level transcription by RNA polymerase III. This requirement for CK2 is clear from the in vivo measurements of pol III transcription in cka2^{ts} mutant where de novo synthesis of tRNAs and 5S rRNA is markedly diminished (Fig. 2-1). The reduced level of pol III transcription, along with the corresponding reduction in pol I transcription, would largely account for diminished levels of overall RNA synthesis in these cells (Hanna et al. 1995).

The in vitro pol III assays further confirm the in vivo results. Thus the reduced CK2 kinase activity in cka2^{ts} extracts is associated with diminished pol III

transcription (Figures 2-2, 2-3). Importantly, the transcription profile of cka2^{ts}, both in vivo and in vitro, demonstrate a specific defect in the synthesis of tRNAs and 5S rRNA primary transcripts (Fig. 2-1, 2-2), thereby indicating impaired initiation of transcription in the CK2 mutant.

Regulation of TFIIIB by phosphorylation

Biochemical fractionation of the pol III transcription machinery from a CK2 mutant demonstrates a specific defect in the basal pol III factor TFIIIB (Fig. 2-7). Conversely, wild type TFIIIB fully restores transcription in CK2-defective extracts (Fig. 2-4, 2-5). These observations suggest that phosphorylation of TFIIIB by CK2 is required for its efficient transcriptional activity. In support of this idea, dephosphorylation of wild type TFIIIB results in a marked loss of its transcriptional activity (Fig. 2-10).

Previous studies using extracts from *Xenopus* eggs suggest that the phosphorylation state of TFIIIB is important for its activity (Hartl et al. 1993; Gottesfeld et al. 1994). For example, the transcriptionally inactive form of TFIIIB from metaphase extracts can be activated by phosphatase treatment. Conversely, the active interphase form of TFIIIB can be inactivated by p34^{cdc2}-cyclin B kinase. These results demonstrate that mitotic phosphorylation can inhibit TFIIIB. The results presented here also indicate a role for phosphorylation in the regulation of TFIIIB, albeit that CK2 phosphorylation activates yeast TFIIIB. p34^{cdc2} and CK2, the known protein kinases implicated in the regulation of TFIIIB, are highly conserved and are expressed in all cycling eukaryotic cells (reviewed in Nurse 1990; Allende and Allende 1995). These observations raise the possibility that the regulation of TFIIIB in eukaryotes is mediated by its differential phosphorylation and that the overall pattern of these events, via phosphorylation of distinct subunits or different residues within a given subunit, determines the transcription activity of TFIIIB. In agreement with this idea, differential phosphorylation of multiple sites that can have distinct and separable effects in regulating the activity of transcription factors has been demonstrated in diverse eukaryotes (Binetruy et al. 1991; Karin and Smeal 1992; Komeili and O'Shea 1999).

The molecular target of CK2 in TFIIIB

The observation that CK2 stimulates transcription in cka2^{ts} extracts suggests a direct role for CK2 in pol III transcription (Fig. 2-9). Moreover, the stimulation of transcription by recombinant TBP implies a specific defect in the transcriptional activity of TBP in CK2-deficient extracts (Fig 2-9). These observations suggest that the positive effect of CK2 in pol III transcription may be mediated by its direct phosphorylation of TBP. Consistent with this hypothesis, recombinant TBP is a CK2 substrate and, among TFIIIB subunits, only TBP is efficiently phosphorylated by CK2 in vitro (Fig. 2-8). The functional significance of this interaction is indicated by the cooperativity of TBP and CK2 in restoring transcription in impaired extracts (Fig. 2-9).

In support of a requirement for phosphorylation of TBP by CK2 in pol III transcription, the S128A substitution of a putative CK2 phosphorylation site in TBP results in impaired transcription in vivo (Fig. 2-12). This finding is in agreement with a previous report in which S128G substitution in yeast TBP impairs transcription in vivo (Cormack and Struhl 1993). Furthermore, the acidic residues that serve as the determinants of the CK2 consensus site S^{128}EDD^{131} in TBP, are highly conserved throughout eukaryotes (Fig. 2-11). Significantly, several point mutations within the consensus domain that result in substitution of the acidic residues with uncharged amino acids also result in impaired in vivo transcription both in yeast and human cells (Cormack and Struhl 1993; Bryant et al. 1996). Taken together, these results provide a working model in which the effect of CK2 in pol III transcription is mediated by its direct phosphorylation of TBP.

Enhanced promoter recruitment of phosphorylated TFIIIB

The gel mobility shift assay reveals that TFIIIB from the CK2-deficient strain displays markedly reduced recruitment to a tRNA^{Tyr} promoter (Fig. 2-14). This deficiency provides a mechanistic basis for the observed defect in transcriptional activity of TFIIIB from these cells (Fig. 2-7). The impaired recruitment of TFIIIB from cka2^{ts} cells is also in agreement with the observed specific defect in the initiation step of pol III transcription in these cells (Figures 2-1 and 2-2). These findings, along

with the impaired recruitment of dephosphorylated TFIIIB (Fig. 2-14), suggest that CK2 phosphorylation is required for efficient promoter recruitment of TFIIIB.

The promoter recruitment of TFIIIB is initiated by its interaction with DNA-bound TFIIIC (Kassavetis et al. 1999). TFIIIB subsequently binds the upstream DNA directly and, in turn, recruits RNA polymerase III enzyme. The observed defect in pre-initiation complex assembly in the $CK2^{ts}$ extract can then be attributed to impaired association of TFIIIB with TFIIIC or reduced intrinsic TFIIIB DNA binding activity. Binding of the Brf1 subunit of TFIIIB to Tfc4, the 131 kDa subunit of TFIIIC that protrudes into the upstream region, largely underlies TFIIIB promoter recruitment by TFIIIC (see schematics in Chapter 1). TBP, the potential CK2 target among TFIIIB subunits, is not implicated in binding of TFIIIB to TFIIIC. On the other hand, TBP footprints to the upstream region (Persinger and Bartholomew 2001), and in fact point mutations of TBP residues E93 and L87 that are in close proximity to DNA result in significant reduction of TBP pol III activity in vitro (Colbert et al. 1998). These findings suggest that impaired promoter recruitment of TFIIIB from the CK2 mutant is likely due to a defect in its DNA binding.

In vitro cross linking experiments indicate that TBP binds the TATA-like sequence TATATGTG which starts 30 nucleotides upstream of the transcription start site of a $tRNA^{Tyr}$ gene (Persinger et al. 1999; Persinger and Bartholomew 2001). These experiments, as well as TBP mutagenesis data (Colbert et al. 1998), suggest that the interactions of TBP with DNA in a TFIIIB-DNA complex are architecturally similar to those in a canonical TBP-TATA box complex (Kim et al. 1993). The crystal structure of TATA-bound yeast TBP reveals that TBP S128 does not contact DNA (Fig. 1-1, Chapter1, p.22). Therefore, the potential CK2 phosphorylation of this residue may alter the conformation of the adjacent DNA binding domains of TBP. This idea, although highly speculative, is not without precedent; phosphorylation of the serum-response transcription factor (SRF) by CK2 on a cluster of four serine residues drastically enhances its DNA binding in vitro (Manak et al. 1990). Partial proteolytic experiments indicate that SRF undergoes a conformational change following its phosphorylation by CK2. Since the phosphorylated residues are not within the minimal DNA binding of the protein, an allosteric mechanism is thought to

mediate the effect of CK2 phosphorylation on SRF DNA binding (Manak and Prywes 1991; Manak and Prywes 1993).

Alternatively, the positive effect of TBP phosphorylation might be displayed through its interactions with other TFIIIB subunits. A genetic screen for yeast TBP mutants that exhibit specific defects in pol III transcription in vivo, has yielded a solvent exposed and nearly continuous surface that is believed to be required for binding of TBP to the Brf1 subunit of TFIIIB (Cormack and Struhl 1993). This surface encompasses the CK2 consensus site within TBP, and in fact TBP E129A mutation is partially suppressed by overexpression of Brf1 (Cormack and Struhl 1993). The Brf1 subunit of TFIIIB extensively interacts with the upstream DNA (Persinger et al. 1999; Persinger and Bartholomew 2001). While the carboxy terminus of Brf1 binds DNA by itself, the full length protein does not. This suggests that the stable DNA binding of Brf1 may be partly attributed to its interaction with TBP (Huet et al. 1996). Whether the CK2 phosphorylation of TBP increases the promoter recruitment of TFIIIB by promoting the DNA binding of its associated Brf1 is not known. Nevertheless, from a mechanistic viewpoint, this idea is similar to the observation that in proliferating human fibroblasts phosphorylation of transcription factor c-Max by CK2 enhances the DNA binding of its obligatory partner c-Myc (Berberich and Cole 1992).

CK2 and the regulatory mechanisms that impinge on TFIIIB

Increasing evidence suggest that TFIIIB is a key target of regulatory mechanisms that govern the output of pol III transcribed genes. In *Xenopus*, the cell cycle regulation of pol III transcription operates at the level of TFIIIB, perhaps through differential phosphorylation of its 92 kDa and TBP subunits (Leresche et al. 1996). The *Drosophila* pol III transcription machinery is sensitive to the cellular concentration of TBP, and in yeast the silencing of transcription in stationary phase is mainly due to a reduced level of Brf1 (Trivedi et al. 1996; Sethy et al. 1995). Furthermore, the strict control of pol III transcription by tumor suppressors is primarily mediated by their regulation of TFIIIB (White et al. 1996; Cairns and White 1998). In summary, TFIIIB is the target of a spectrum of regulatory effects that can act on distinct subunits of TFIIIB. The data presented here demonstrate that the

positive effect of CK2 in pol III transcription in yeast is also mediated via its regulation of TFIIIB. CK2 is conserved and ubiquitously expressed in eukaryotes, as are TFIIIB subunits. Furthermore, the promoter organization of pol III genes, and the role of TFIIIB in transcription of these genes, is remarkably similar among eukaryotes (Huang and Maria 2001; Paule and White 2000). These observations suggest that CK2-mediated upregulation of TFIIIB may be universally employed throughout eukaryotes.

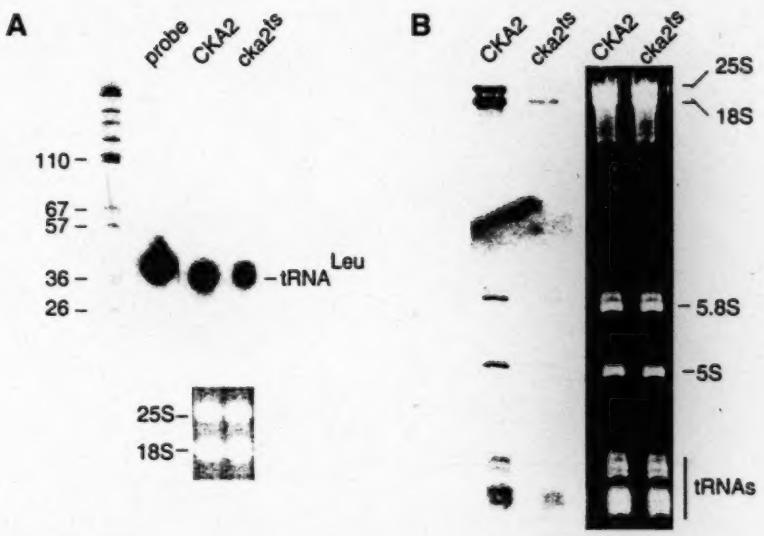


Figure 2-1. In vivo transcription by RNA polymerase III is impaired in a CK2 mutant. A. Total RNA prepared from CKA2 and cka2^{ts} cells grown at the permissive temperature were probed for the expression of tRNA^{Leu} by an S1 nuclease protection assay. The undigested DNA probe and the DNA molecular weight markers are also shown. Equivalent RNA recovery was confirmed by electrophoresing 5µg of each total RNA sample in a 1% formaldehyde-agarose gel and staining with ethidium bromide to detect the large rRNAs (lower panel). **B.** Analysis of the in vivo labelled RNAs in cells grown at the permissive temperature. After 30 min of labeling with ^{3}H-uracil, total RNA was prepared and 10µg aliquots were resolved by denaturing PAGE. The gel was stained with ethidium bromide, photographed (right panel), and then processed for fluorography (left panel).

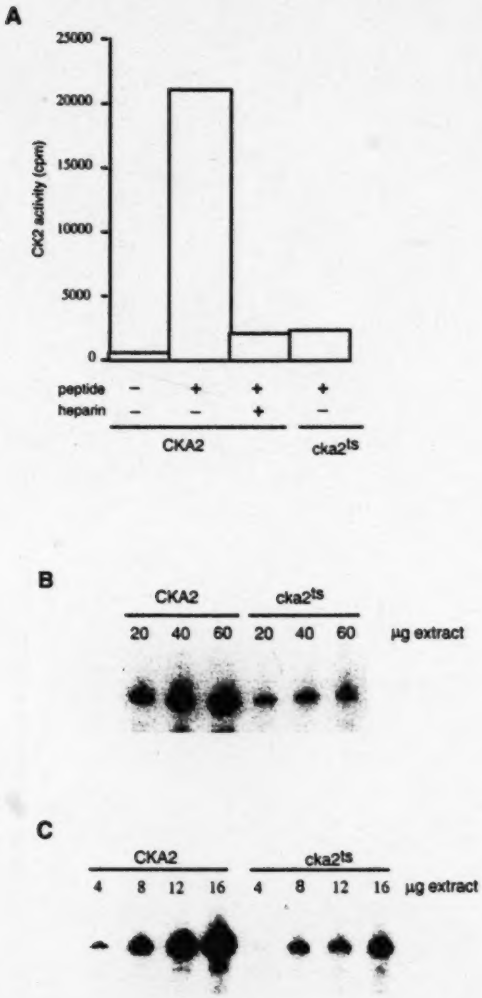


Figure 2-2. Transcription by RNA polymerase III is deficient in whole cell and nuclear extracts from a CK2 mutant. **A.** CK2 activity, determined by phosphorylation of a CK2 substrate peptide, is deficient in whole cell extract (6µg) prepared from cka2^{ts} strain grown at the permissive temperature. When indicated, heparin (2 µg/ml) was used as a CK2 inhibitor. **B.** In vitro transcription of 5S rRNA (400 ng/reaction) is impaired in the CK2-deficient whole cell extract. The in vitro transcribed RNA are resolved by a denaturing gel and detected by autoradiography. The primary (unprocessed) 5S rRNA transcripts are shown. **C.** Pol III transcription is impaired in nuclear extract from the CK2 mutant grown at the permissive temperature. Panel as in **B**.

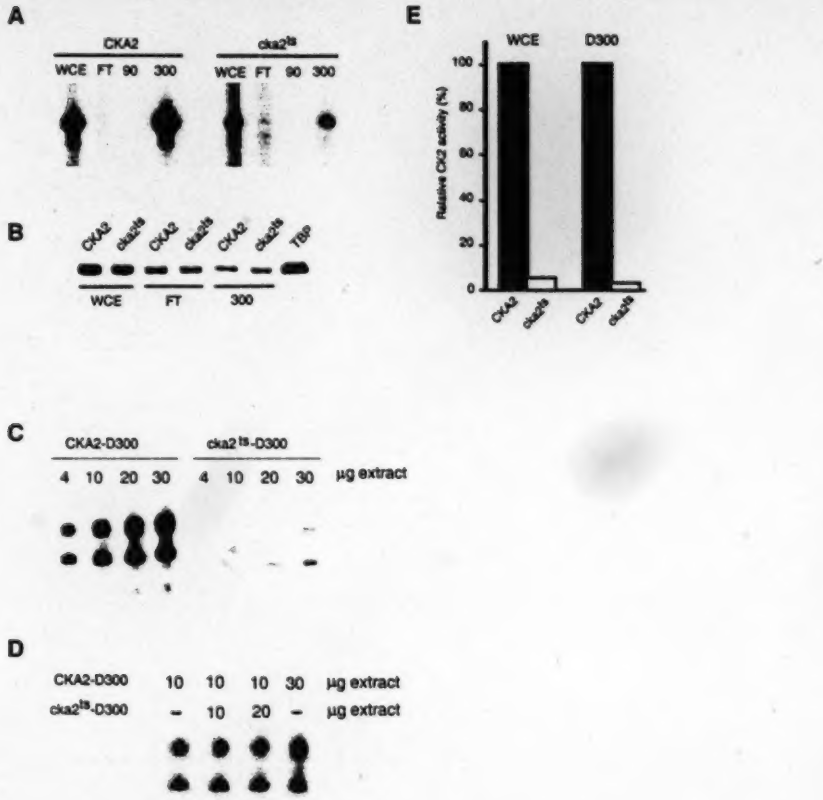


Figure 2-3. The transcriptional defect in CK2-deficient extract is preserved after partial fractionation of the pol III transcription factors. A. Whole cell extracts (WCE) prepared from the wild type (CKA2) and CK2-deficient ($cka2^{ts}$) strains grown at the permissive temperature were chromatgraphed in parallel on DEAE resins. The flow-through fractions (FT) were collected and the columns were washed extensively with a buffer containing 90 mM KCl (90). The pol III factors were eluted at 300 mM KCl. Equal µg of the collected fractions from each strain were assayed for in vitro transcription of a 5S rRNA template (400 ng/reaction). The RNA products were resolved by electrophoresis on a denaturing gel and detected by autoradiography. **B.** Equal amounts of DEAE fractions from each strain were monitored for TFIIIB content by Western blotting against TBP. Purified recombinant yeast TBP was loaded in the last lane. **C.** The difference in transcriptional activity of D300 fractions from wild type and CK2-deficient extracts is maintained over a broad range of protein concentration. The indicated amounts of each fraction were assayed for in vitro transcription of a $tRNA^{Tyr}$ template (20 ng/reaction). The upper and the lower bands denote the primary (unprocessed) and 5' end-processed tRNA transcripts, respectively. **D.** Impaired transcription in CK2-deficient extract is not due to a dominant repressor. The indicated amounts of D300 fractions from wild type (CKA2) and CK2 mutant ($cka2^{ts}$) grown at the permissive temperature were mixed prior to addition of $tRNA^{Tyr}$ template. The in vitro transcribed RNA were resolved by a denaturing polyacrymide gel and detected by autoradiogrphy. **E.** The difference in bulk CK2 activity in CK2 and $cka2^{ts}$ whole cell extracts (WCE) is preserved in the corresponding D300 fractions.

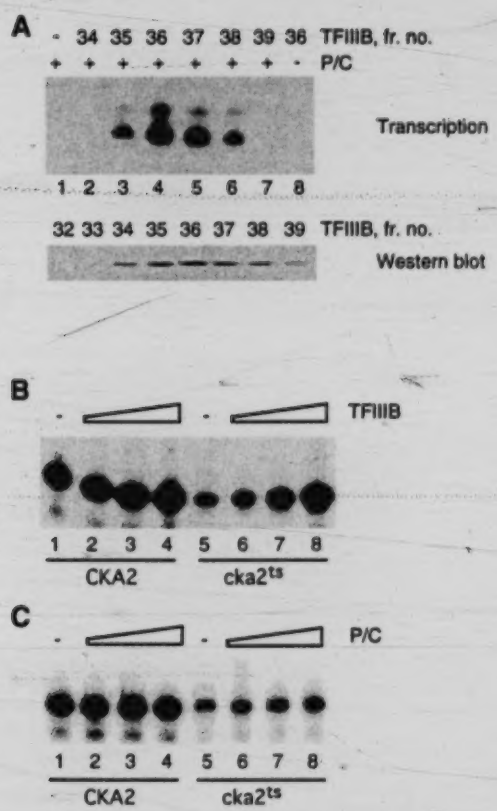

A
- 34 35 36 37 38 39 36 TFIIIB, fr. no.
+ + + + + + + - P/C
Transcription
1 2 3 4 5 6 7 8
32 33 34 35 36 37 38 39 TFIIIB, fr. no.
Western blot
B
- - TFIIIB
1 2 3 4 5 6 7 8
CKA2
cka2ts
C
- - P/C
1 2 3 4 5 6 7 8
CKA2
cka2ts

Figure 2-4. Pol III transcription in CK2-deficient whole cell extract is rescued by TFIIIB. A. Top panel. The P/C (RNA polymerase III and TFIIIC) and TFIIIB fractions reconstitute pol III transcription (see Fig. 6-1, Chapter 6). $tRNA^{Tyr}$ transcription (20 ng template/reaction) was assayed using 4µl of P/C, either added to 10µl aliquots of fractions from the hydroxyapatite column used to purify TFIIIB (lanes 1-7), or on its own (lane 8). Bottom panel. The peak of TFIIIB activity obtained by hydroxyapatite chromatography corresponds to the peak of TBP. Immunoblot of TFIIIB fractions (12µl aliquots) using antiserum raised against recombinant TBP. **B.** TFIIIB (0, 0.4, 1 and 2.5µl) slightly stimulates 5S rRNA transcription in CKA2 extract (lanes 1-4) and fully restores transcription in $cka2^{ts}$ extract (lanes 5-8). **C.** The P/C fraction (0, 0.8, 2 and 5µl) does not significantly stimulate 5S rRNA transcription in CKA2 or $cka2^{ts}$ extract. The reactions in **B** and **C** used 60µg of extract and 400 ng of plasmid pY5S.

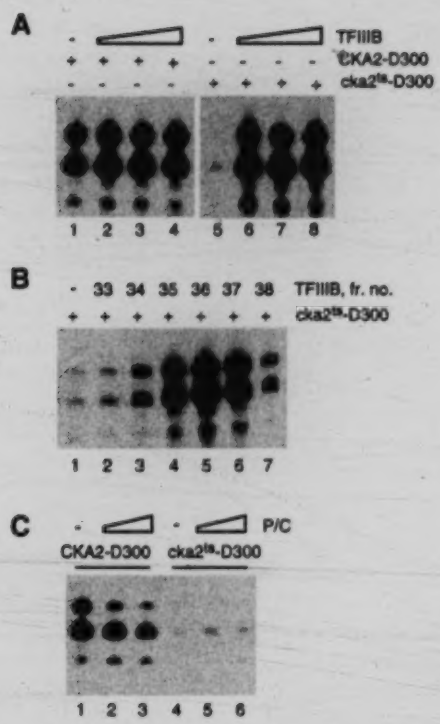


Figure 2-5. TFIIIB restores transcription in the 300 mM KCl DEAE (D-300) fraction of defective extract. A. TFIIIB marginally stimulates tRNA^{Tyr} transcription in CKA2-D300 (lanes 1-4) and fully restores transcription in cka2^{ts}-D300 (lanes 5-8). The reactions received 0, 2, 5 or 10µl hydroxyapatite TFIIIB and 13 µg D300 fraction. **B.** The peak of TFIIIB activity (fraction 36, Fig. 2-4, PanelA) corresponds to the peak of activity that restores transcription in cka2^{ts}-D300 (lane 5). Transcription was assayed using 13 µg cka2^{ts}-D300 added to 10µl aliquots of fractions from the hydroxyapatite column used to purify TFIIIB. The template was at 20 ng/reaction. **C.** The P/C fraction inhibits tRNA transcription in the D300 fraction from CKA2 extract (lanes 1-4) and only marginally stimulates transcription in the corresponding fraction from cka2^{ts} extract

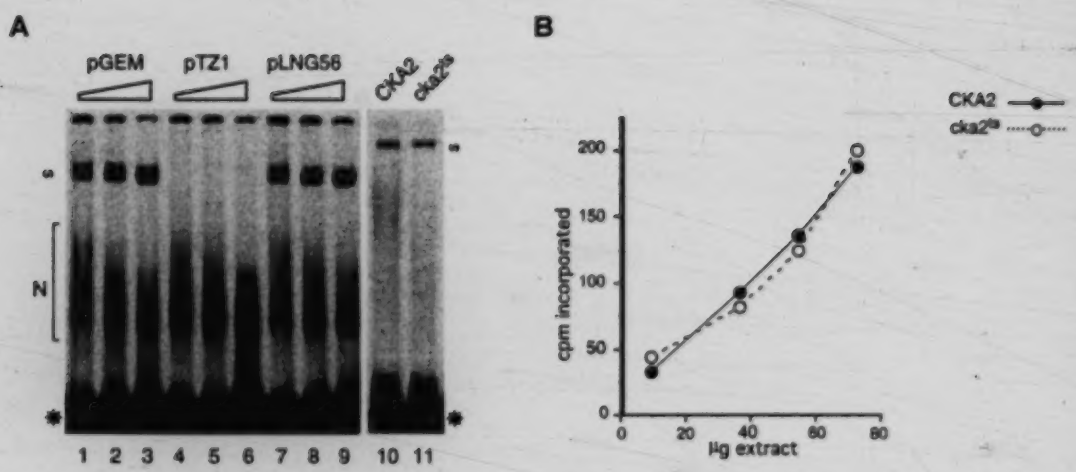


Figure 2-6. TFIIIC and RNA polymerase III are equally active in wild type and cka2^{ts} mutant. A. TFIIIC DNA binding activity in the D300 fraction of wild type extract (3.5µg ; lanes 1-9). The probe was an end-labeled restriction fragment from pTZ1. Specificity of binding was established using increasing amounts (700, 1000, 1700 ng) of non-specific competitor (pGEM3), specific competitor (pTZ1), or a binding site mutant competitor (pLNG56). Lanes 10 and 11 compare TFIIIC binding in the D300 fractions from CKA2 and cka2^{ts} cells. The specific TFIIIC shift (-) and free probe (*) are indicated. A variable amount of non-specific binding to probe DNA was observed in all experiments (N). **B.** Bulk polymerase III activity in CKA2 and cka2^{ts} whole cell extracts. Bulk polymerase activity is expressed as counts per min (cpm) incorporated during a 25 min reaction at room temperature.

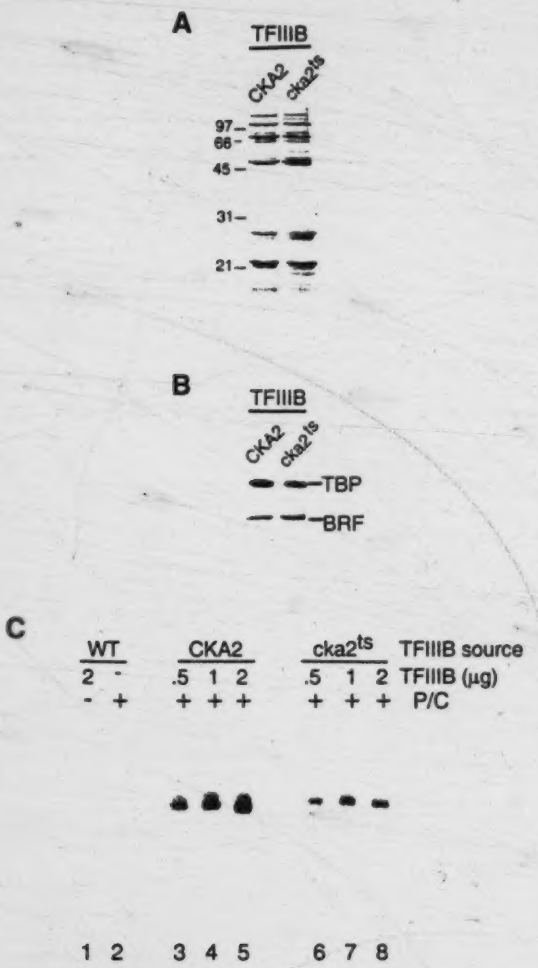


Figure 2-7. TFIIIB from CK2-deficient cells is transcriptionally impaired. A. The protein profile of TFIIIB isolated in parallel from wild type (CKA2) and cka2^{ts} cells. 10 µg of each fraction was electrophoresed on a 10% SDS-polyacrylamide gel and silver-stained. The positions of the protein molecular weight markers are indicated on the left. **B.** Equal amounts of TFIIIB from the wild type and the CK2 mutant were assayed for TBP and Brf content by Western blotting. **C.** TFIIIB from CK2-deficient strain is impaired in reconstitution of pol III transcription in vitro. Reactions contained 2.4µg of a pol III/TFIIIC (P/C) fraction and the indicated amounts of Cibacron Blue fractionated TFIIIB. ^{32}P-UTP-labeled tRNA^{Tyr} primary transcripts were resolved in a denaturing polyacrylamide gel and detected by autoradiography (Panel as in Fig. 2-4).

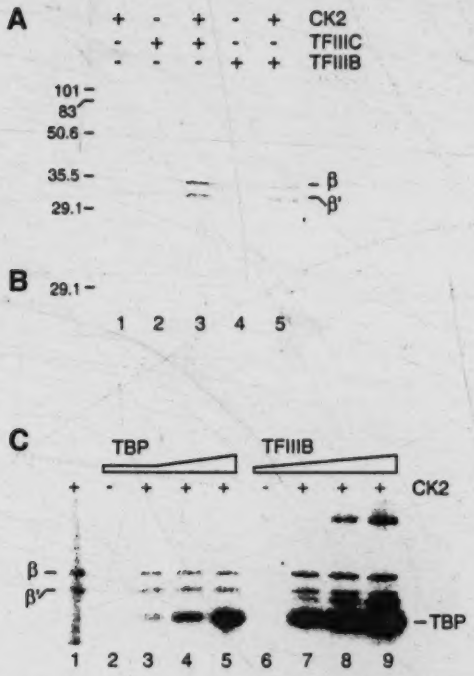


Figure 2-8. The TBP subunit of TFIIIB is preferentially phosphorylated by CK2. A. Autoradiograph showing the labeled products resulting from in vitro phosphorylation of cellular TFIIIB (Cibacron Blue fraction, 10 µl) and affinity purified TFIIIC (10 µl) with purified yeast CK2 (5 µl). The positions of the molecular weight markers and the autophosphorylated β and β' subunits of CK2 are shown on the left and right, respectively. γ-^{32}P-GTP was used as the phosphate donor. **B.** Western blot analysis of the products in panel **A** using a polyclonal antiserum raised against TBP. As judged by the position of the markers, the immunoreactive band in lanes 4 and 5 comigrates with the labeled 29 kDa band in **A**, lane 5. **C.** Recombinant TBP is phosphorylated by CK2 in vitro. In vitro kinase assays were performed with γ-^{32}P-ATP as the phosphate donor. Reactions contained CK2 on its own (lane 1), TBP on its own (lane 2), CK2 plus increasing amounts of recombinant TBP (0.1, 0.2, 0.5 µl; lanes 3-5), TFIIIB on its own (Cibacron Blue fraction, 12 µl; lane 6), and CK2 plus increasing amounts of TFIIIB (4, 8, 12 µl; lanes 7-9). Endogenous protein kinase(s) present in TFIIIB phosphorylate a number of proteins (white dots) that are only detected in longer exposures (compare with panel **A**, lane 4).

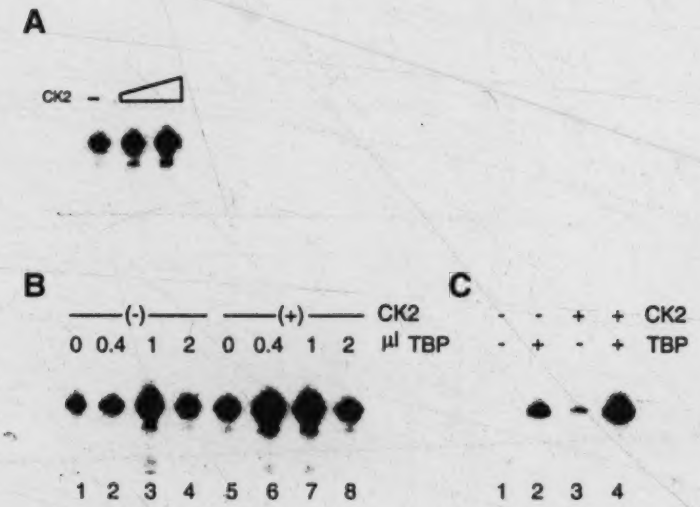


Figure 2-9. Recombinant TBP and a limiting amount of CK2 rescues tRNA transcription in CK2-deficient extract. A. Purified yeast CK2 (0.1 and 0.5 µl) partially stimulates 5S rRNA transcription in nuclear extract (16 µg) from cka2^{ts} cells grown at the permissive temperature. **B.** Nuclear extract (16 µg) from cka2^{ts} cells was supplemented with TBP alone (lanes 1-4) or increasing amounts of TBP in the presence of CK2 (0.5 µl, lanes 6-8). **C.** The D300 fraction (11 µg) from cka2^{ts} cells was supplemented with buffer (lane 1), TBP (2 µl, lane 2), CK2 (2.5 µl, lane 3), or TBP plus CK2 (lane 4). Plasmid pY5S was used as the template at 400 ng/reaction.

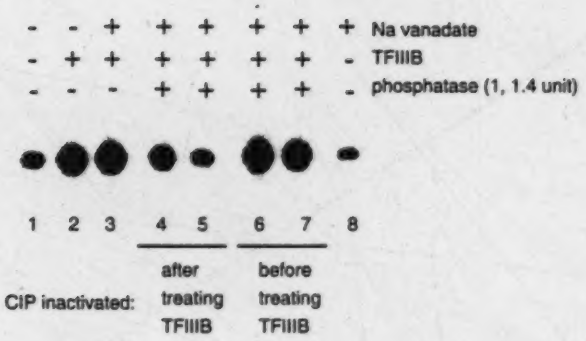
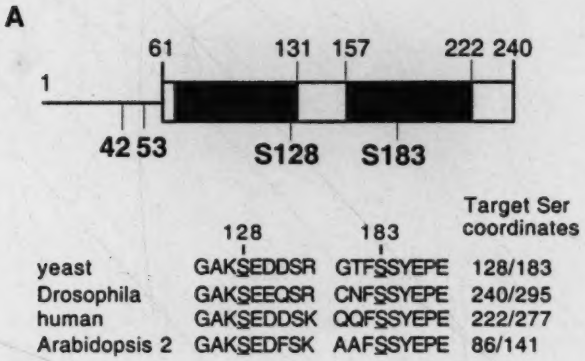


Figure 2-10. TFIIIB must be phosphorylated in order to restore transcription in cka2^{ts} extract. TFIIIB was treated with alkaline phosphatase (CIP) in the presence or absence of sodium vanadate, then added to cka2^{ts} nuclear transcription extract. The final composition of each reaction is indicated in the panel above the autoradiograph and the timing of CIP inactivation (by addition of sodium vanadate) is indicated below the lane numbers. TFIIIB retained its capacity to stimulate 5S rRNA transcription in cka2^{ts} extract when incubated with phosphatase and sodium vanadate together (compare lanes 2, 3 with 6, 7), but was unable to stimulate cka2^{ts} extract when sodium vanadate was added after phosphatase treatment (compare lanes 3, 7 with 5). Sodium vanadate on its own has little effect on transcription (lane 8).



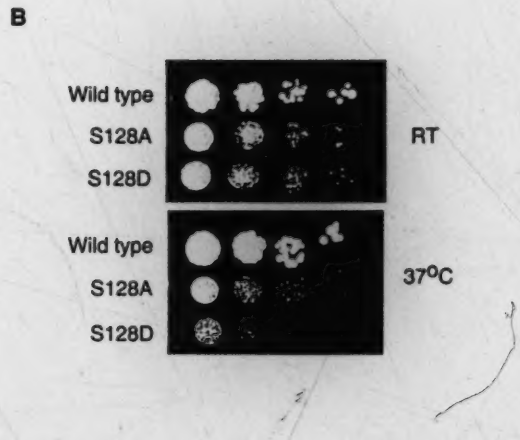


Figure 2-11. Potential CK2 sites in TBP. A. Location of potential CK2 sites (bold) in the essential C-terminus (box) of yeast TBP. This essential domain spans the residues 61-240 and includes a repeated motif (filled regions of the box) that is involved in binding of TBP to the TATA box of pol II promoters. (Lower panel) potential CK2 sites (shaded) in yeast TBP and the sequence of the corresponding sites in three metazoan species, *Drosophila*, human, and *Arabidopsis* (from TBP sequence alignment in (Nikolov et al., 1992)). **B.** 10-fold serial dilutions of the strains harboring wild type, S128A, or S128D allele of TBP grown for 3 days at room temperature or 37°C.

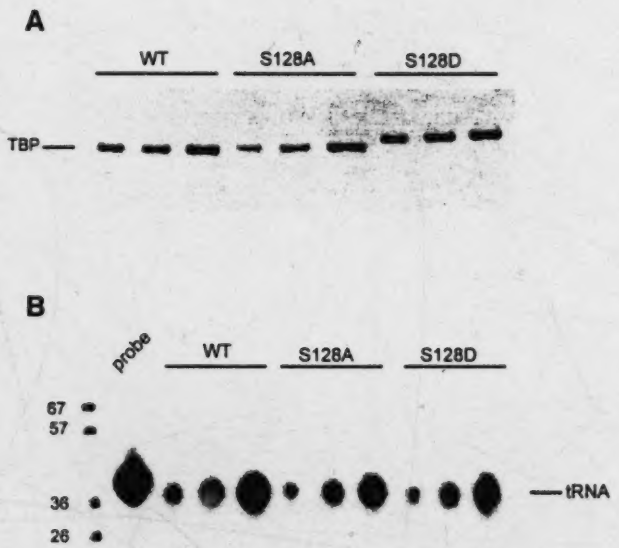


Figure 2-12. The effects of TBP S128 mutations in pol III transcription. A. TBP expression in S128A, S128D, and wild type strains. 5, 10, and 20μg of whole cell extracts were assayed for TBP content by immunoblotting. **B.** Impaired in vivo pol III transcription in TBP mutants. Increasing amounts (5, 10, 20μg) of total RNA from each strain were assayed by a S1 nuclease protection assay. The position of DNA size markers and undigested DNA probe is depicted on the left. The cells were heat shocked for 90 min at 37°C prior to preparation of extracts and RNA.

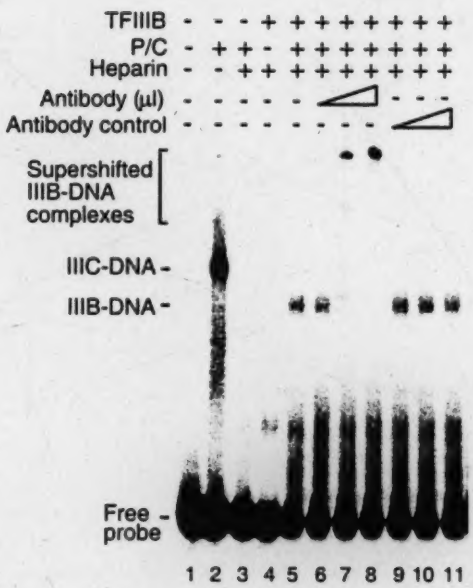

TFIIIB - - - + + + + + + + +
P/C - + + - + + + + + + +
Heparin - - + + + + + + + + +
Antibody (µl) - - - - - - - - - -
Antibody control - - - - - - - - - -
Supershifted IIIB-DNA complexes
IIIC-DNA
IIIB-DNA
Free probe
1 2 3 4 5 6 7 8 9 10 11

Figure 2-13. Formation of a stable TFIIIB-DNA complex on a tRNA^{Tyr} promoter. Binding reactions were performed using 0.5µg pol III/TFIIIC (P/C) and 0.6µg TFIIIB. Supershifting using anti-TBP antibodies (0.01, 0.04, 0.2µl, lanes 6-8) affinity-purified from immune serum and control IgGs from preimmune serum (0.01, 0.04, 0.2µl lanes 9-11) confirmed formation of the IIIB-DNA complex. Note the disruption of IIIC-DNA complex by heparin (lanes 2 and 3).

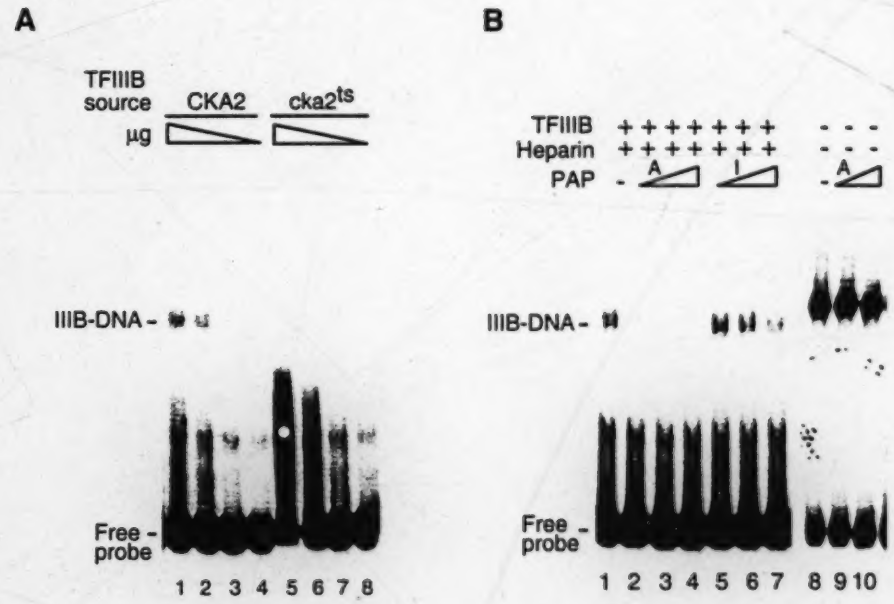


Figure 2-14. CK2 controls promoter recruitment of TFIIIB. A. TFIIIB from CK2-deficient cells does not form the TFIIIB-DNA complex as efficiently as wild type TFIIIB. The reactions used 0.6, 0.3, 0.15 and 0.08 µg of TFIIIB in the presence of 150µg/ml heparin. The broad smear in lane 5 (dot) was not observed in all experiments. **B.** Dephosphorylation of TFIIIB impairs its ability to form a stable complex with promoter DNA. 0.6 µg TFIIIB was pretreated with potato acid phosphatase (PAP, 0.1, 0.2, 0.4µg/ ml A[ctive]), or co-incubated with PAP and phosphatase inhibitor (PAP, I[nactive]). PAP and its inhibitor do not affect DNA binding by TFIIIC (lanes 8-10).

Chapter 3

Association of catalytically active CK2 with TFIIIB

Parts of this chapter have been published:

Ghavidel, A., D. J. Hockman and M. C. Schultz (1999). "A review of progress towards elucidating the role of protein kinase CK2 in polymerase III transcription: regulation of the TATA binding protein." Mol Cell Biochem **191**(1-2): 143-148.

Ghavidel, A., M.C. Schultz (1997). "Casein kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein." Genes Dev. **11**: 2780-2789.

Introduction

The interactions of CK2 subunits with their respective partners result in constitutive and stable binding of CK2 to many of its substrates in vivo (reviewed in Glover 1998; Guerra et al. 1998). The sequence alignment of CK2β reveals that 48 residues are identical in diverse eukaryotes. The crystal structure of the human CK2β indicates that these residues form surfaces that are located around the entire CK2β dimer (Chantalat et al. 1999). The majority of these residues are not implicated in CK2β dimerization or assembly of the holoenzyme, and should be available as binding sites for interacting proteins (Niefind et al. 2001; Grein et al. 1999). Proteins that interact with CK2 are localized to diverse cellular compartments. Binding of CK2 to these proteins may in fact underlie its broad subcellular localization (Lebrin, et al. 1999; Bosc et al. 2000; also reviewed in Faust 2000).

In proliferating mammalian cells, subsets of cellular CK2 are localized to the nucleus (Lorenz et al. 1993; Filhol et al. 1994) and the nucleolus (Belenguer et al. 1989). Subcellular fractionation indicates that a population of CK2 is also nuclear in *S. cerevisiae* (Fig. 4-6, Chapter 4, p 95). Given its regulation of TFIIIB, the possibility that CK2 may be constitutively associated with TFIIIB was examined. It is shown here that in *S. cerevisiae*, CK2 cofractionates with TFIIIB and coimmunoprecipitates with TBP. The kinase activity of the TBP-associated CK2 is greatly reduced in a CK2β null strain, suggesting that the β subunit is required for proper assembly or efficient activity of the TBP-CK2 complex. Consistent with the requirement for CK2 kinase activity in efficient pol I and pol III transcription, the in vivo synthesis of tRNA and rRNA is impaired in the CK2β null strain.

Results

CK2 is associated with TFIIIB

In order to examine the possible association of CK2 with TFIIIB, the chromatographic profile of TBP, Brf1, and CK2β during TFIIIB purification was monitored by immunoblotting (Fig. 3-1). Chromosomal *ckb1Δ* strains expressing GST or GST-tagged CK2β in a plasmid vector were used for this experiment. GST-β, but not GST alone, rescues the salt-sensitivity phenotype in these cells, thus indicating that CK2β tagged with GST is not functionally compromised (personal observation). As expected from the various functions attributed to CK2, its broad subcellular localization, and the diversity of its protein-protein interactions (Glover 1998; Guerra and Issinger 1999), the bulk of CK2β and casein kinase activity fractionates away from TFIIIB. However, on the hydroxylapatite resin (see Fig. 6-1, p113 for a schematic of TFIIIB fractionation) a small fraction of GST-β precisely co-fractionates with TBP and Brf1 (Fig. 3-1, A, the ratio of this GST-β relative to total cellular pool was not determined) and TFIIIB activity (Fig. 3-1, B). Although the chromatographic profile of TFIIIB is apparently similar between strains that express GST or GST-β, GST alone does not co-fractionate with TFIIIB on hydroxylapatite (Fig. 3-1, A, lane 13), indicating that the association of GST-β with TFIIIB is mediated by β. Quantitative immunoblotting yields a molar ratio of 1:0.4:0.4 for TBP, Brf1, and GST-β in hydroxylapatite fraction 5 which exhibits the highest transcriptional activity. Similar to Brf1 and GST-β, some functional components of other TBP-containing complexes have also been recovered in non-stoichiometric ratios relative to TBP (Comai et al. 1992; Poon et al. 1995). This may be due to dissociation of these proteins from their respective complexes throughout chromatography. Nevertheless, the 1:1 stoichiometry of Brf1 and GST-β and impaired in vivo transcription in a CK2β null strain (Fig. 3-6) suggest that the recovery of CK2β with TFIIIB is reflective of its function in pol III transcription.

Consistent with the in vivo association of the α and β subunits, a kinase activity that phosphorylates casein also cofractionates with TFIIIB and CK2β (Fig. 3-1, C). This activity is also present in TFIIIB from *cka1Δ CKA2* cells that had been additionally chromatographed on Cibacron Blue (Fig. 3-2, lane 1). Biochemical characterization of this kinase reveals that it utilizes GTP, is inhibited by a peptide

used to assay conventional CK2, and is highly sensitive to heparin, a classical CK2 inhibitor (Fig. 3-2, lanes 4-7). These biochemical properties indicate that phosphotransferase activity that cofractionates with TFIIIB is likely due to CK2. This idea is supported by the observation that TFIIIB-associated kinase activity is greatly diminished in TFIIIB from isogenic *cka1*Δcka2^{ts} cells (Fig. 3-2, lane 2). These results indicate that a subpopulation of active CK2 molecules is associated with chromatographically purified TFIIIB.

Further evidence for association of CK2 with TFIIIB was sought by probing TBP immune complexes for the presence of CK2 subunits. Lysates were prepared from wild type yeast strains that, in addition to the endogenous CK2 subunits, express GST, or GST-tagged versions of α', β, or β'. Immunoblotting of lysates reveals similar expression levels of the tagged CK2 subunits (Fig. 3-3, lanes 2-4). GST-β, GST-β' and GST-α' are readily detectable in TBP immune complexes, while GST alone, despite higher expression level (lane 1), is not (Fig. 3-3, lanes 5-10).

A TBP-associated, GTP-dependent kinase that can phosphorylate exogenous casein (Fig. 3-4, A, lanes 4, 5) is also detectable in TBP immune complexes from wild type (*CKA2*) cells. However, this kinase activity is greatly reduced in cka2^{ts} cells (Fig 3-4, A, lanes 6, 7). Similar to the TFIIIB-associated kinase, the TBP-associated kinase is highly sensitive to competitor peptide substrate and heparin (Fig. 3-4, lanes 8-11). Importantly, the kinase activity also phosphorylates endogenous and recombinant TBP, the likely CK2 substrate among TFIIIB subunits (Fig. 3-4, B). These data demonstrate that enzymatically active CK2 is associated with TBP.

Regulation of the TBP-associated CK2 by the β subunit

CK2 regulatory subunits directly interact with some substrates and in part dictate the enzyme's substrate specificity in vitro (Grein et al. 1999; Guerra et al. 1999). Since both yeast CK2 regulatory subunits are recovered with TBP, it is possible that association of CK2 with TBP is mediated by direct interaction of these subunits with TBP. Therefore, interaction of the in vitro-labeled β and β' with TBP was examined. Significant retention of β, but not β', on a TBP-affinity matrix was evident (Fig. 3-5, A, lanes 4-9). Furthermore, the activity of TBP-associated CK2 is substantially diminished in CK2β null cells (Fig. 3-5, B, 1-3). These results suggest

an important role for β in regulation of the TBP-associated CK2. In support of this idea, in vivo pol I and pol III transcription is impaired in a CK2β null strain (Fig. 3-6).

Discussion

It was shown in the preceding chapter that CK2 functionally interacts with TFIIIB. The results presented in this chapter demonstrate that this interaction occurs via association of CK2 with TFIIIB. This association is evidently mediated by direct interaction of the kinase with the TBP subunit. Thus CK2β binds TBP and CK2 phosphorylates TBP in vitro. Importantly, direct phosphorylation of TBP by CK2 provides a plausible mechanism for the coordinated regulation of pol I and pol III transcription by CK2 (Fig. 2-1, p 42, Fig. 3-6).

Cofractionation of CK2 with TFIIIB suggests that TFIIIB exists as a pre-assembled CK2-containing complex. This organization is similar to that of TFIID, the TFIIIB counterpart in pol II transcription, which contains basal transcription factors in complex with pol II accessory proteins (Poon et al. 1995; Moqtaderi et al. 1996). The stable association of CK2 with TFIIIB further implies that a sub-population of the cellular CK2 is functionally dedicated to pol III transcription. Given the similar requirement for CK2 in pol I and pol III transcription in yeast and the presence of the TBP-containing pol I core transcription factor SL1 as a pre-assembled complex (Moehle and Hinnebusch 1991), there may also exists a pol I-dedicated population of CK2.

Regulation of the TBP associated-CK2 by CK2β

The activity of the TBP-associated CK2 is reduced in a CK2βΔ strain (Fig. 3-5). Consistent with a requirement for TBP phosphorylation in efficient transcription, the in vivo synthesis of tRNA and rRNA is also impaired in these cells (Fig. 3-6). CK2β increases the catalytic activity of the kinase toward most substrates in vitro (Allende and Allende 1998). Therefore, the reduced kinase activity of the TBP associated-CK2 in the ckb1Δ strain may be due to a requirement for the β subunit in efficient phosphorylation of TBP by the catalytic subunits. In fact recombinant *Xenopus* CK2β stimulates the phosphorylation of TBP by the catalytic subunit (Maldonado and Allende 1999). While this effect may be due to allosteric

activation of the CK2α, it is equally plausible that binding of CK2β to TBP increases the net recruitment of the catalytic subunit to the TBP substrate by, for example, enhancing the stability of the TBP-CK2 complex. Comparing the in vivo transcription profile of the cka2^{n} cells to that of an isogenic β null strain indicates that the deleterious effect of the CK2βdisruption in pol I and pol III transcription is less profound than that of an inactivating mutation in the catalytic subunit (Fig. 3-6). An interpretation of this finding is that CK2α alone interacts with TBP in vivo and the stability of this complex requires CK2β. This idea is in agreement with the observation that CK2β can directly bind TBP in vitro (Fig. 3-5). Similarly, CK2β mediates the association of the CK2 holoenzyme with p53 and cell surface receptor CD5 (Raman et al. 1998; Gotz et al. 1999). While the architectural organization of CK2-TBP complex awaits structural studies, the collective data indicate a role for CK2β in regulation of TBP-associated CK2, and by extension, in pol I and pol III transcription.

Formation of the TBP-CK2 complex may occur via electrostatic interactions

The overall similarity of the deduced TFIIIB-DNA complex organization to the structure of the canonical TATA-bound TBP suggests that, while in complex with other TFIIIB subunits, extensive surface areas of TBP are available for docking of interacting partners (Persinger et al. 1999). Perhaps the most striking structural feature of the DNA-bound TBP is the highly basic convex surface of the TBP saddle that can provide for electrostatic interactions with acidic region of interacting proteins (Fig. 1-1, Chapter 1). Importantly, TBP undergoes no obvious conformational changes after binding to DNA (Kim et al. 1993). Therefore, TBP residues not implicated in DNA-binding or interaction with other TFIIIB subunits, should be available for binding of CK2 whether TFIIIB is DNA-bound or not. This provides an explanation for the apparently constitutive association of CK2 with TBP (Fig. 3-1).

Conversely, the internal acidic domain of human CK2β is solvent exposed and has been implicated in binding to p53 and p21^{WAF1} (Niefind et al. 2001). This domain, which is not involved in CK2β dimerization or holoenzyme assembly, is conserved in yeast CK2β and provides an attractive site for possible electrostatic interactions with the basic surface of TBP. However, despite conservation of this

domain in both CK2β and CK2β', only CK2β detectably binds TBP in vitro (Fig. 3-5). It may be that the acidic region of CK2β is not involved in binding to TBP. Alternatively, the interaction of this domain with the basic surface of TBP may be stabilized by non-conserved residues unique to CK2β. This idea is not without precedence since in vitro binding of $p21^{WAF1}$ requires short segments within both carboxy and N-terminal domains of human CK2β in addition to the acidic domain (Gotz et al. 2000).

Given the tight correlation between protein synthesis and cellular proliferation rate (Polymenis and Schmidt 1997), the apparently wild type growth phenotype of strains that carry either of the catalytic subunits suggest that α and α' have overlapping functions in pol III transcription. Furthermore, the recovery of both CK2β and CK2β' in TBP immunocomplexes indicate that both regulatory subunits can associate with TBP. While CK2β clearly regulates the activity of TBP-associated kinase, the functional significance of CK2β' in pol III transcription remains unknown.

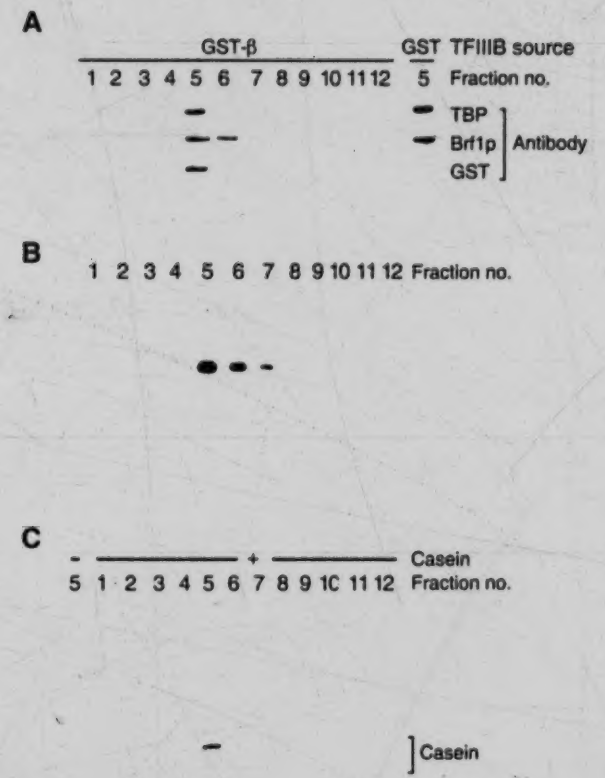


Figure 3-1. Association of active CK2 with TFIIIB. A. Co-purification of CK2 with TFIIIB. Fractions from the hydroxylapatite step of TFIIIB purification were monitored for TFIIIB and CK2β by immunoblotting using the indicated antibodies; for a particular antibody the same volume of each fraction was analyzed. Expression of GST-tagged CK2β (lanes 1-12) or GST alone (lane 13) in cells grown in CM-Ura medium was induced with 0.6 mM $CuSO_4$ for 1 hr prior to harvesting. **B.** Reconstitution of $tRNA^{Tyr}$ transcription using hydroxylapatite fractions from cells expressing GST-β (panel **A**). Reactions contained 2.4 μg of a pol III/TFIIIC fraction (P/C) from wild type cells and 2μl of each hydroxylapatite fraction. ^{32}P-UTP-labeled $tRNA^{Tyr}$ gene products were resolved in a denaturing polyacrylamide gel and detected by autoradiography. **C.** CK2 activity was assayed in hydroxylapatite fractions (0.5 μl aliquots) from cells expressing GST-CK2β. Assays were performed with 40μg/ ml casein as the exogenous substrate and [γ-^{32}P]GTP as the phosphate donor.

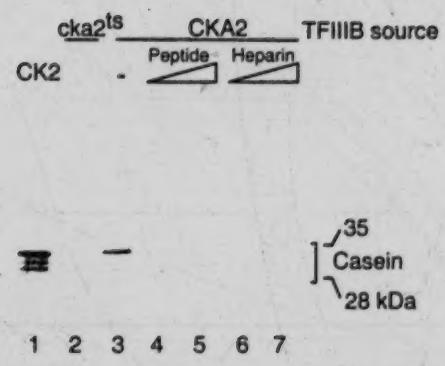


Figure 3-2. Biochemical characterization of the TFIIIB-associated kinase. CK2 activity is associated with Cibacron Blue TFIIIB from CKA2 but not cka2^{ts} cells. In vitro phosphorylation of casein, as the exogenous substrate of the TFIIIB-associated kinase was conducted as in Fig. 3-1. When indicated, CK2 substrate peptide (1 and 4 μg/μl) and heparin (2 and 6 ng/μl) were used as inhibitors of CK2.

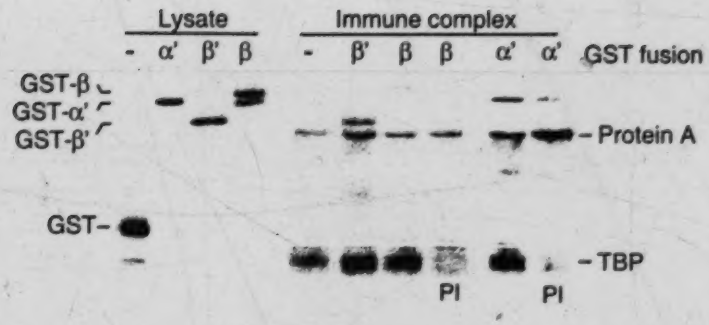
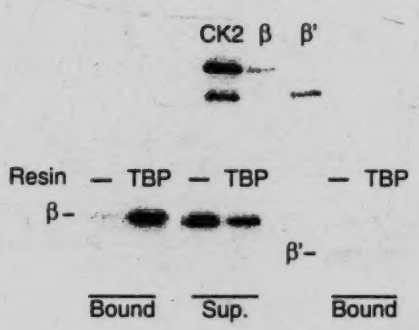


Figure 3-3. CK2 subunits are associated with TBP in vivo. CK2 subunits coimmunoprecipitate with TBP. Lysates (4 μg) and TBP immune complexes from cells expressing the indicated GST fusion constructs, or GST alone (lanes 1, 5), were analyzed by immunoblotting using anti-GST or anti-TBP antibody. Pre-immune serum was used for immunoprecipitation in lanes 8 and 10, where the smear in the vicinity of TBP is IgG light chain. A detectable amount of GST-α' is non-specifically recovered in lane 10 owing to its interaction with Protein A-Sepharose. The protein in GST-β lysates (lane 4) labeled with an asterisk may be a breakdown product of GST-β.

Figure 3-4. A TBP-associated protein kinase with the biochemical properties of CK2. A. TBP was immunoprecipitated from lysates of the indicated strains and assayed for casein (40, 120μg/ ml) kinase activity. Where indicated, the immunoprecipitated complexes were incubated with CK2 peptide (1, 3 mg/ml) or heparin (0.8, 2 μg/ ml) for 5 min prior to the addition of casein and ^{32}P-GTP. Labeled proteins were detected by autoradiography and TBP recovery was monitored by immunoblotting (lower panel). Pre-immune serum was used in lane 1. **B.** Recombinant TBP is a substrate of TBP-associated CK2. Purified recombinant yeast TBP (40, 120 μg/ml) was added to TBP immune complex from CKA2 cells. The labeling reaction was performed as in **A**.



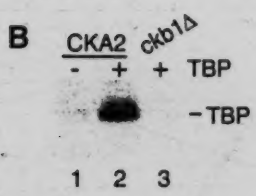


Figure 3-5. Direct physical interaction of CK2β with TBP in vitro. A. CK2 was autophosphorylated (lane 1) and β and β' recovered by preparative SDS-PAGE. The isolated subunits were analyzed by SDS-PAGE (lanes 2, 3) and chromatographed on BSA or TBP affinity resins (lanes 4-9); bound and unbound (supernatant) proteins were detected by autoradiography. **B.** CK2 activity in TBP immune complexes is repressed in cells that do not express CK2β (strain ckb1Δ, YAPB6 of Bidwai et al., 1995). TBP immune complexes were assayed for their ability to phosphorylate 120 μg/ml of added recombinant TBP as in Fig. 3-4.

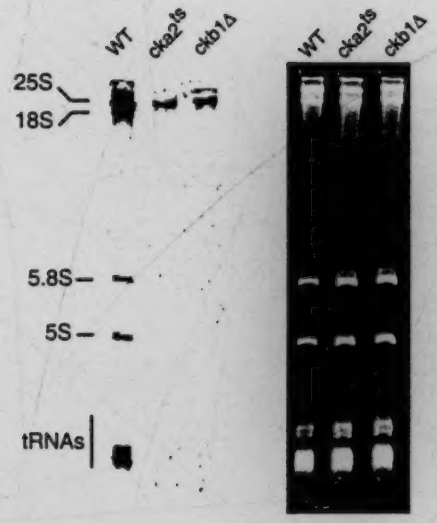


Figure 3-6. Impaired pol III transcription in CK2 mutants. A temperature sensitive strain of CK2 ($cka2^{ts}$), a CK2β null mutant (ckb1Δ), and their isogenic wild type (WT) were grown at 27°C and labelled with ^{3}H-uracil, for 30 min. 10μg of the in vivo labelled RNA from each strain was resolved by denaturing PAGE. The gel was stained with ethidium bromide to determine loading (right panel) and then processed for flurography (left panel).

Chapter 4

Repression of RNA polymerase III transcription in response to DNA damage via TBP-associated CK2

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Introduction

Formation of DNA lesions is a constant occurrence in the cell (reviewed in Wang 1998; Weinert 1998; Zhou and Elledge 2000). Sources of DNA damage may be extrinsic or intrinsic to the cell. Extrinsic sources of DNA damage include natural UV and ionizing radiation, as well as chemical mutagens. Normal cellular events such as DNA replication and recombination generate strand breaks and thus can serve as intrinsic sources of DNA damage; single and double strand breaks are generated by the nicking and closing activity of DNA topoisomerases which are required to remove torsional stress ahead of the replication forks (Lamb et al. 1993). Base mismatches arise by occasional incorporation of wrong base during replication and may go undetected by the mismatch repair system. Double strand breaks also occur as regulated components of cellular events such as meiosis (Lamb et al. 1989; Wilkie et al. 1990). Moreover, byproducts of cellular metabolism give rise to reactive species such as free radicals that can covalently modify or crosslink DNA bases (Siddiqi and Bothe 1987; Bothe et al. 1990).

If DNA damage remains unrepaired, the initial damage can be changed into secondary lesions as cells progress through the cell cycle. For example, when a replication fork encounters a covalently modified base, as in a pyrimidine dimer, it may resume replication downstream of the damage (Kuzminov 1995). This results in formation of a daughter strand gap that encompasses the damage. Replication of this gapped DNA in the subsequent S phase results in formation of a double strand break that may result in chromosomal truncation and gene loss or alternatively, in chromosomal rearrangement due to the highly recombinogenic nature of double strand breaks (Garvik et al. 1995). The deleterious effects of unrepaired double strand breaks are also manifested in mitosis in which the centrosome-containing and acentric fragments may be partitioned into separate nuclei (Keil and Roeder 1984; Voelkel-Meiman et al. 1987). Moreover, DNA replication across lesions in the template strand, a process called translesion synthesis, often results in incorporation of noncognate bases in the nascent strand which may be fixed during subsequent round of replication (Kuzminov 1995). Similarly, replication of naturally occurring

mismatch bases results in fixation of these mutations in one of the daughter duplexes (Paulovich et al. 1997).

Genomic mutations are proposed to serve as a mechanism for natural selection. Thus the high mutation rate in cancer cells allows for a greater rate of genomic evolution (Paulovich et al. 1997). The same selective pressure might occur during the evolution of organisms when rapid changes in genome are advantageous (Dianov et al. 2001). Nevertheless, the deleterious effects of accumulated mutations necessitate cellular mechanisms that maintain genomic stability. Accordingly, cells possess multiple surveillance mechanisms that delay cell cycle progression when damage to the genome is detected. These surveillance mechanisms are collectively termed checkpoint controls.

DNA damage checkpoints

DNA damage checkpoints were initially identified by genetic studies in *S. cerevisiae* which yielded loss of function mutations that relieved the dependence of cell cycle progression on completion of DNA replication or repair (Weinert and Hartwell 1988; Hartwell and Weinert 1989; Weinert et al. 1994). These responses are found in all eukaryotic cells examined and are mediated by products of highly conserved genes (reviewed in Wang 1998; Birrell et al. 2001). Checkpoints are functionally defined as signal transduction pathways that communicate information between DNA lesions and the cell cycle machinery (Fig. 4-1). Checkpoint-mediated cell cycle arrest is thought to allow cells time to repair DNA before progression into the cell cycle. Similar to other signaling pathways, DNA damage checkpoints are mediated by components that can be grouped into three broad classes of sensors, signal transducers, and effectors (Weinert 1998; Zhou and Elledge 2000).

DNA damage sensors and checkpoint activation

The available data suggest that checkpoint activation is initiated by processing of DNA lesions into signaling-competent secondary lesions (Lydall and Weinert 1995; Lydall and Weinert 1997). This may explain how cells respond to structurally diverse DNA lesions by employing a limited repertoire of signaling components. Single strand DNA may be one of the secondary lesions that activate the DNA damage

checkpoints. Alkylated DNA and UV-induced photoproducts such as pyrimidine dimers are initially converted to double strand breaks during S phase and subsequently processed into single strand DNA by cellular exonucleases (Weinert et al. 1994; Garvik et al. 1995; Navas et al. 1996). The link between DNA damage processing and cell cycle arrest has precedence in the bacterial SOS response. In *Escherichia coli*, single strand DNA generated from processing of double strand breaks by recBC helicase/exonuclease complex precedes induction of the SOS response (Zhou and Elledge 2000).

Human Mre11 is part of an evolutionarily conserved multisubunit NBS1 nuclease complex that also contains Nbs1 and Rad50 proteins (Johzuka and Ogawa 1995; Ogawa et al. 1995). While the precise function of NBS1 complex in processing DNA lesions remains unclear, it displays several properties that are consistent with a role as an initiator of DNA damage-mediated signaling; Mre11 localizes to sites of DNA lesions and its 3'-5' nuclease activity is required for the formation of single strand DNA at these sites (Usui et al. 1998; Nelms et al. 1998). Moreover, Nbs1 protein is phosphorylated in response to DNA damage and this phosphorylation is required for induction of S phase arrest (Furuse et al. 1998). Accordingly, hypomorphic mutations in *MRE11* or *NBS1* in human cells are associated with DNA damage sensitivity and S phase checkpoint deficiency (Usui et al. 1998). Deletion of Xrs2 protein, the counterpart of Nbs1 in yeast, also results in similar phenotypes (D'Amours and Jackson 2001). Mutations in the Nbs1 subunit that impair S phase checkpoint initiation result in the genetic disorder Nijmegen breakage syndrome that is associated with increased predisposition to cancer (Petrini 2000).

Yeast single strand binding protein RPA is thought to bind to the generated single strand DNA (Lee et al. 1998). RPA is a heterotrimeric protein complex that destabilizes the DNA double helix during replication, thereby permitting the parental DNA strands to serve as template (Boubnov and Weaver 1995). The evidence in support of a role for RPA in DNA damage-induced signaling events comes from its role in regulating adaptation in *S. cerevisiae*. Induction of an irrepairable double strand break in yeast results in a prolonged (8-10 hours) but transient arrest at G2/M (Toczyski et al. 1997; Lee et al. 1998). Following adaptation, cells grow and divide for several generations before they lose viability, presumably due to progressive

degradation of the broken chromosome that eventually results in loss of essential genes. Mutation in the large subunit of RPA suppresses G2/M arrest in cells with irrepairable double strand breaks (Lee et al. 1998). Consistent with the role of Mre11 in generating the single strand DNA, deletion of Mre11 similarly suppresses G2/M arrest.

Genetic studies in yeast have yielded additional genes whose concerted functions are specifically required for DNA damage-mediated G2/M arrest. In *S. cerevisiae,* replication factor C (RFC) consists of one large and four small subunits and is required for DNA replication in normal growth (Sugimoto et al. 1997; Shimomura et al. 1998). RFC is a structure-specific DNA binding protein complex that recognizes the primer-template junction. It recruits proliferating cell nuclear antigen (PCNA) which forms a sliding clamp that tethers DNA polymerase to the template. After induction of DNA damage, yeast RFC complex is recruited to the sites of the DNA lesions by an unknown mechanism (Naiki et al. 2000). RFC, in turn, recruits a heterotrimeric complex of Rad17/Ddc1/Mec3. Modeling studies suggest that these proteins, similar to PCNA, may form a doughnut-like structure that could be loaded onto damaged DNA just as PCNA is loaded onto primed DNA (Thelen et al. 1999; Venclovas and Thelen 2000). Sequence homology reveals that Rad17 encodes a putative 3'-5' DNA exonuclease. This suggests that the generation of single strand DNA, similar to its role in induction of S phase checkpoint, may also serve as the initiating signal for G2/M arrest (Shimada et al. 1999).

Signal transducers and effectors of the checkpoints

The sensor proteins, in turn, activate the proximal signal transducers encoded by *MEC1* and *TEL1* genes in yeast (Canman et al. 1998). Sequence homology indicates that these kinases may be functionally related to the phosphatidyl inositol-3 kinases. Mutations in ATM, the human counterpart of yeast Mec1, is associated with the disorder ataxia telangiectasia that is associated with immune deficiency and high frequency of cancer (Huang et al. 1998; Zhou and Elledge 2000). Cells from these patients, similar to yeast *mec1*Δ mutant, exhibit defects in cell cycle arrest at G2/M and S phase, are impaired in transcriptional induction of genes that encode DNA

repair proteins, and display marked sensitivity to DNA damaging agents (Sanchez et al. 1997).

The role of signal transducer attributed to these kinases is largely because deletions or inactivating mutations in the kinase domain abolish the phosphorylation of several downstream effectors (Lydall and Weinert 1995; Lydall and Weinert 1997; Sanchez et al. 1997). Among their targets are human Chk1 and Chk2 kinases. These serine/threonine kinases are structurally unrelated but share some overlapping substrates in vitro (Melchionna et al. 2000). Chk2 is phosphorylated in response to DNA damage and this phosphorylation is apparently required for its activation since dephosphorylated Chk2 displays reduced kinase activity in vitro (Matsuoka et al. 1998). While ATM phosphorylates Chk2 in response to ionizing radiation, phosphorylation of Chk2 after UV irradiation and replication block requires ATR, the human homolog of yeast Tel1 (Matsuoka et al. 2000). *CHK2*$^{-/-}$ thymocytes treated with ionizing radiation do not stabilize or activate p53 and, therefore, fail to transcribe p53-inducible genes, or trigger apoptosis (Chehab et al. 2000; Hirao et al. 2000). The p53 tumor suppressor is a key regulator of the cellular responses to genotoxic stress (reviewed in Lohrum and Vousden 2000; Vousden 2000). It has a very short half life since it is normally degraded by a ubiquitination-dependent mechanism that requires ubiquitin ligase Mdm2 (Fang et al. 2000). In irradiated cells, Mdm2 undergoes rapid ATM/Chk2 dependent phosphorylation and this event is concomitant with an increase in p53 protein stability (Ryan et al. 2000). p53 induces the expression of p21, a potent inhibitor of cyclin dependent kinase B, that results in G1/S arrest (Khosravi et al. 1999; Maya et al. 2001). Genotoxic stress in *S. cerevisiae* also results in G1/S arrest, albeit by a different mechanism; DNA damage results in Mec1-dependent phosphorylation of Rad53, the Chk2 homolog in yeast (Sanchez et al. 1997). Activated Rad53 induces the expression of transcriptional regulator Swi6 which in turn inhibits the expression of Swi4 that is required for transcription of G1 cyclins (Sidorova and Breeden 1997).

The induction of G2/M arrest in both mammals and yeast is partly mediated by maintaining the inhibitory phosphorylation of p34$^{CDC2/CDC28}$ (Weinert 1998). Dephosphorylation and subsequent activation of this key regulator of the cell cycle is catalyzed by the dual specificity phosphatase Cdc25 (Surana et al. 1993). In cells with

DNA damage, Cdc25 becomes phosphorylated by Chk2 protein kinase. This phosphorylation is thought to maintain the inhibitory S-phase specific phosphorylation of Cdc25 which prevents Cdc25 from activating $p34^{CDC2}$ (Yang et al. 1999). Accordingly, $p34^{CDC2}$ Y15F mutant remains active in cell cycle progression, but is defective in G2/M arrest (Sorger and Murray 1992; Lew and Reed 1995).

An additional mechanism for DNA-damage induced M-phase arrest has been described in yeast; in normal growth, Pds1, a protein that maintains sister chromatid cohesion, is proteolytically degraded before initiation of anaphase (Cohen-Fix et al. 1996). Moreover, exit from mitosis requires the inactivation of the mitiotic cyclin-dependent kinase Cdc28 by destruction of the M phase-specific cyclins. Pds1 inhibits cyclin destruction, thus ensuring that mitotic exit is delayed until after completion of anaphase (Cohen-Fix and Koshland 1997). Importantly, DNA damage results in Mec1-dependent phosphorylation of Pds1 which renders it resistant to ubiquitin-dependent proteolysis. The stabilization of Pds1 provides a mechanism that delays both anaphase initiation and mitotic exit in cells with DNA damage.

Aside from the induction of cell cycle arrest, checkpoint activation is coordinated with changes in diverse cellular processes. For instance, cells with DNA damage drastically reduce the rate of DNA replication since ongoing replication can render mutations permanent. This is accomplished by inhibiting the firing of replication origins and reducing the rate of DNA polymerase elongation (Painter et al. 1987; Larner et al. 1997). Other processes that are regulated in response to checkpoint activation, such as chromatin reconfiguration at the sites of the DNA lesions and global changes in cellular transcription are discussed below.

DNA damage and chromatin assembly

The eukaryotic genome is compacted within a nucleoprotein complex known as chromatin, the basic units of which are nucleosome core particles that are composed of histones (Wolffe 2000). Nucleosomes normally restrict access to DNA and as such their dynamic rearrangement precedes fundamental cellular processes such as nuclear transcription and recombination (Cairns 1998). Several studies have demonstrated that the repair of damaged DNA in human and yeast cell-free extracts is inhibited in reconstituted nucleosomes (Wang et al. 1991; Wang et al. 1993; Ura et al.

2001). Importantly, addition of recombinant chromatin remodeling complex ACF enhances the repair of UV-induced pyrimidine dimers by human nucleotide excision repair in vitro (Ura et al. 2001). ACF is among several ATP-dependent chromatin remodeling factors that are implicated in facilitating transcription of nucleosomal DNA (reviewed in Kingston and Narlikar 1999; Aalfs et al. 2001). A requirement for chromatin reconfiguration at the site of DNA lesions is supported by studies in patients with Cockayne syndrome (CS), a genetic disorder that is associated with high sensitivity to UV (Nance and Berry 1992). The *CSB* gene encodes a nuclear protein which shows sequence homology to the SWI/SNF family of chromatin remodeling factors (Eisen et al. 1995). CSB directly binds histones and exhibits chromatin remodeling activity in vitro (Citterio et al. 2000). Importantly, CSB interacts with RNA polymerase II both in vivo and in vitro (Van Gool et al. 1997; Vermeulen et al. 1997). It is proposed that CSB, by binding to stalled RNA polymerase II, is recruited to DNA lesions where it remodels chromatin and thus facilitates DNA access by the repair machinery (Citteiro et al. 2000). This role is consistent with the observed defect in transcription-coupled repair in CS patients (see below). Spatial correlation between DNA repair and chromatin remodeling is also suggested by the observation that yeast SWI/SNF chromatin remodeling complex contains Rad16 and Rad54 that are required for nucleotide excision repair and repair by homologous recombination, respectively (Eisen et al. 1995).

After completion of DNA repair, the preexisting organization of the chromatin needs to be restored. Restoration of chromatin organization would be especially critical in regions of the genome where a precise chromatin structure is essential to maintaining transcriptional regulation. Two recent reports provide evidence for possible mechanistic coupling of DNA repair and chromatin assembly (Emili et al. 2001; Hu et al. 2001). Anti-silencing factor (Asf1) was originally identified in a *S. cerevisiae* overexpression screen as a suppressor of the telomeric transcriptional silencing (Singer et al. 1998). The *Drosophila* homolog of yeast Asf1 is a histone chaperone that participates in nucleosome assembly onto de novo synthesized DNA in vitro (Tyler et al. 1999). Deletion of yeast *ASF1* confers sensitivity to MMS and replication block (Emili et al. 2001; Hu et al. 2001). Conversely, overexpression of Asf1 in yeast partially restores the viability of a *rad53* mutant after replication block.

In addition to their genetic interaction, Rad53 and Asf1 proteins are in a complex in unperturbed cells. However, Asf1 dissociates from Rad53 in cells with DNA damage and this event requires Mec1, the upstream activator of Rad53 (Hu et al. 2001). It is thought that Asf1 takes part in chromatin assembly onto newly repaired DNA. Coordinated DNA repair and assembly is also observed in human cell-free extracts in which repair and assembly of preexisting DNA lesions occur with similar kinetics (Kaufman et al. 1997; Gaillard et al. 1999).

The yeast silent information regulator (Sir2-4) complex is required for maintaining transcriptional repression at the telomeres and the silent mating loci (Grunstein 1997). It is thought that they repress transcription by polymerizing across the nucleosomes and creating a transcriptionally inactive heterochromatin state. Induction of DNA damage in *S. cerevisiae* results in striking redistribution of the Sir3 and Sir4 proteins from telomeres to sites of DNA lesions in a Mec1-dependent manner (Martin et al. 1999; Mills et al. 1999). The Ku70/Ku80 complex that is essential for double strand break repair by non-homologous DNA end joining apparently recruits the Sir proteins to these sites (Kanaar et al. 1998; Dronkert et al. 2000). In support of a physiological relevance for this event, deletion of either *SIR* genes results in MMS sensitivity (Mills et al. 1999).

Collectively, these data point to a model in which chromatin reconfiguration by the SWI/SNF family of proteins at the sites of DNA damage precedes binding of the repair machinery. It is thought that the structural intermediates, such as single strand DNA generated by processing of DNA lesions, are shielded from spurious recombination by binding of the Sir proteins (Mills et al. 1999). After completion of DNA repair, nucleosomes are reconfigured to their original state by the recruited chromatin assembly factors. Restoration of chromatin structure can in principle signal the completion of the repair process and resumption of the cell cycle.

Transcription-coupled DNA damage repair

The initial evidence that DNA repair and transcription may be mechanistically coupled came from the observation in Chinese hamster ovary cells in which pyrimidine dimers in the actively transcribed DHFR gene were repaired nearly five times faster compared to the flanking non-transcribed region of the genome (Bohr et

al. 1985). Subsequent studies have demonstrated that the transcribed strand is repaired significantly faster than the non-transcribed strand (Bowman et al. 1997; Smith et al. 2000).

A potential mechanism for coupling transcription and DNA repair is provided by the complementation studies in patients with the genetic disorder xeroderma pigmentosum (XP) (Drapkin et al. 1994; Tong et al. 1995; LeRoy et al. 1998). The defect in DNA repair in XP cells underlies the increased propensity to cancer among XP patients. These cells are specifically impaired in nucleotide excision repair (NER), a process that repairs many structurally diverse DNA lesions (Houtsmuller et al. 1999; also reviwed in Drapkin et al. 1994). NER requires the coordinated action of several helicases and at least two exonucleases that excise nearly 30 bp of DNA that encompass the lesion. Several of the human genes involved in NER, designated excision repair cross complement (ERCC) genes, were initially identified by correcting NER in XP cells (Huang et al. 1992; Matsunaga et al. 1995; Zamble et al. 1996; Khan et al. 1998). Surprisingly, two of these genes encode human helicases ERCC2 and ERCC3 that are subunits of TFIIH, a pol II basal transcription factor that is required for phosphorylation of the carboxy terminal domain of RNA polymerase II and its subsequent promoter clearance (Guzder et al. 1997). In *S. cerevisiae*, TFIIH is comprised of seven essential subunits that include two helicases that are highly homologous to mammalian ERCC2 and ERCC3 proteins (Matsunaga et al. 1995; Park et al. 1995; Reardon et al. 1997). Extracts from conditional mutants of each of these subunits are defective in NER, thereby suggesting the involvement of holo-TFIIH complex in DNA repair (Thompson et al. 1994; Guzder et al. 1997). TFIIH is not normally associated with elongating RNA polymerase II complex. However, in vitro competition studies demonstrate that addition of damaged DNA results in significant dissociation of TFIIH from a pol II promoter which, in turn, results in inhibition of pol II transcription (You et al. 1998). Importantly, inhibition of transcription in the presence of active NER requires Rad26, the yeast homolog of the human Cockayne syndrome B that is implicated in chromatin remodeling at the site of stalled RNA polymerase (Citterio et al. 2000). These findings suggest that TFIIH, by virtue of its interaction with Rad26, may be preferentially recruited to the stalled RNA polymerase where it participates in NER. Although verification of this idea awaits in vivo studies,

it is nevertheless of obvious potential utility since recruitment of TFIIH not only results in template strand repair, but also limits synthesis of mutated or truncated RNA from damaged templates.

Unlike the preferential repair of actively transcribed pol II genes, repair of pol I and pol III transcribed genes lacks gene specificity (Vos and Wauthier 1991; Stevnsner et al. 1993; Aboussekhra and Thoma 1998). Analysis of DNA isolated after UV irradiation of human fibroblasts indicates that repair of the pol II transcribed *JUN* is completed nearly four times faster than pol III transcribed $tRNA^{Val}$ coding region (Dammann and Pfeifer 1997). It is thought that the pol III transcription factor TFIIIC which footprints most of a 100-nucleotide long tRNA gene, along with the high density of transcribing RNA polymerases on these genes, sterically occlude the DNA repair machinery. In vitro studies demonstrate that binding of pol III transcription factor TFIIIA can significantly reduce the removal rate of UV-induced photoproducts from a 5SrRNA promoter by NER (Conconi et al. 1999). Unlike most pol II transcribed genes, genes that encode rRNAs and tRNAs exist in multiple copies throughout the genome. Therefore, the physiological consequence of delayed repair of these genes is likely to be less severe.

Regulation of cellular transcription after DNA damage

DNA damage induces robust expression of diverse genes that are involved in cellular DNA repair process (reviewed in Longhese et al. 1998; Weinert 1998). The expression of these genes requires functionally intact checkpoints, thus indicating that their transcriptional induction is a part of the global cellular response to DNA damage. The best studied among these genes are the genes that encode a family of ribonucleotide reductase proteins RNR1-4.

These proteins mediate the conversion of ribonucleoside diphosphate to deoxynucleoside triphosphate which serve as precursors in DNA replication (Huang et al. 1997; Huang et al. 1998). In normal growth these genes are transcriptionally repressed because of the binding of Crt1 transcription repressor to the X box, a 13-nucleotide upstream promoter element common to all these genes. Crt1 is phosphorylated in response to DNA damage and replication block and this phosphorylation requires intact Mec1/Rad53 pathway (Huang et al. 1998). The

phosphorylation-mediated dissociation of Crt1 coordinately derepresses the expression of RNR genes. In fact derepression of this regulon by deletion of *CRT1* largely suppresses the DNA damage sensitivity of the hypomorphic *mec1* and *rad53* mutations (Cho et al. 2001). The *RNR* promoters contain multiple X boxes of different strengths. This could potentially allow for a graded response according to the extent of DNA damage or duration of an inducing signal (Huang et al. 1998).

Checkpoint-mediated transcriptional regulation of genes with known functions in cell cycle progression may partly underlie the execution of the cell cycle arrest. Transcription from the *CDC2* promoter is repressed after gamma and UV irradiation (Azzam et al. 1997; De Toledo et al. 1998; Garrett et al. 2001). In cells with DNA damage, the transcriptional repressor CDF-1 binds to a bipartite upstream element and represses the expression of Cdc2 mRNA via an unknown mechanism. Importantly, the same bipartite element is also present in the promoter region of cyclin B1 and *CDC25* genes and is required for their transcriptional downregulation by CDF-1 (Azzam et al. 1997; De tolido et al. 1998). The coordinated repression of these genes may play a role in induction of the G2/M arrest in irradiated cells.

Recent genome wide studies using DNA microarrays in *S. cerevisiae* indicate that the scope of changes in cellular transcription after DNA damage is considerably greater than previously thought (Jelinsky and Samson 1999; Jelinsky et al. 2000; Gasch et al. 2001). Expression of >500 mRNA species is altered after treatment of yeast cells with the DNA alkylating agent MMS and ionizing radiation. Surprisingly, expression of the vast majority of these genes is similarly altered by environmental stress response (ESR) pathways that are activated by general environmental perturbations such as heat shock, osmotic stress, and amino acid starvation (Gasch et al. 2000). The products of these genes are implicated in maintaining cellular homeostasis by regulating protein folding and metabolism, RNA processing, and membrane biogenesis. The correlation in cellular RNA expression in response to stress and DNA damage suggests that ESR activation is a component of the overall cellular response to DNA damage (see Chapter 5 for a more detailed discussion).

DNA damage and pol I/pol III transcription

Eukaryotic pol I and pol III transcription are strictly regulated in response to diverse physiological cues (reviewed in Warner 1999; Geiduschek and Kassavetis 2001). Despite extensive studies of DNA damage-mediated changes in mRNA expression, the regulation of tRNA and rRNA synthesis after DNA damage is poorly understood. Two early reports provided the first insights into regulation of these processes after DNA damage (Feldman 1977; Eliceiri 1979); experiments designed to study the organization of the transcription units unexpectedly revealed that accumulation of low molecular weight cellular RNAs, that were not transcribed by pol II, was reduced after UV irradiation. Since this effect was unlikely due to premature termination of transcription, it was postulated that UV irradiation had resulted in reduced synthesis of these RNA species (Eliceiri 1979).

The results presented in this chapter confirm and extend these earlier findings. It is shown that in vivo pol I and pol III transcription in *Saccharomyces cerevisiae* is impaired in cells that have experienced genotoxic stress. The in vivo defect in pol III transcription is maintained in extracts prepared from cells with DNA damage. This defect is due to impaired initiation and is entirely rescued by addition of the basal pol III transcription factor TFIIIB from untreated cells. The DNA damage-mediated repression of pol I and pol III transcription is largely governed via regulation of TBP-associated CK2. Accordingly, CK2 and S128A TBP mutations effectively abolish the transcriptional response after DNA damage. The coordinated repression of pol I and pol III transcription may play a role in induction of the cell cycle arrest and enhancement of viability in cells with DNA damage.

Results

Repression of pol I and pol III transcription in response to DNA damage

In order to examine the level of pol I and pol III transcription, yeast cells were treated with methane methylsulfonate (MMS), a DNA alkylating agent that is widely used to study the DNA damage response. MMS strongly represses pol I and pol III transcription in vivo, as measured by metabolic labeling with ^{3}H-uracil and S1

nuclease protection analysis (Fig. 4-2, A, B). The latter assay ensures that the observed effect is not due to impaired uptake or processing of the labeled uracil after MMS treatment. Synthesis of tRNA and rRNA is also repressed at UV exposures known to induce the DNA damage response genes in yeast (Fig. 4-2, C; Aboussekhra et al. 1996). Importantly, *cdc* mutants arrested in G2/M (*cdc15-2*) and G1 (*cdc28-1*) maintain a fold of transcriptional repression that is similar to that of the wild type cells (Fig. 4-3, A). This indicates that the transcriptional repression is not an indirect effect of cell cycle arrest that is induced after genotoxic stress.

TFIIIB is the target of a DNA damage response pathway

In order to examine the effect of MMS on pol III transcription in vitro, extracts from control and MMS-treated cells were used to transcribe an exogenous template. Transcription directed by a tRNA gene was inhibited in a dose-dependent manner in whole cell extract and a chromatographic fraction from MMS-treated cells (Fig. 4-3, B, C). Damage of the template DNA is therefore unlikely to directly account for the repression of transcription in vivo. Transcriptional downregulation is evidently not due to differential recovery of components of the pol III machinery from MMS-treated cells since the concentrations of TBP and Brf1 were similar between matched extracts or fractions (Fig. 4-3, B, C, lower panels).

Fractionated pol III factors from untreated cells were separately added to MMS-treated extract in an attempt to reconstitute transcription. TFIIIB fully restored transcription in MMS-treated extract whereas a fraction containing pol III and TFIIIC (P/C) does not (Fig. 4-4, A, B). This indicates that TFIIIB is specifically downregulated in response to DNA damage. Since a single purified factor fully restores transcription in MMS-treated extract, the observed transcriptional repression in vivo in response to genotoxic stress is not a result of general cytolysis.

Protein kinase CK2 has been implicated in diverse physiological responses (reviewed in Glover 1998; Guerra et al. 1999). As a regulator of TFIIIB, CK2 is therefore in a position to modulate tRNA/5S rRNA synthesis according to the physiological status of the cell. Consistent with the idea that a CK2-dependent effect on TFIIIB underlies the downregulation of transcription by MMS, purified CK2 holoenzyme stimulated tRNA transcription in MMS-treated extracts (Fig. 4-4, C).

However, even in larger amounts than shown, CK2 had only a modest effect compared to TFIIIB. Similarly, CK2 only partially restores pol III transcription in extracts from a cka2^{ts} strain (Fig. 2-10, Chapter 2, p 51). This may be due to the apparent inactivation of CK2 activity as a result of self-aggregation upon its dilution to physiological salt concentrations from high-salt storage buffer (Zandomeni et al. 1986; Miyata and Yahara, 1992, A.G. unpublished observation).

TBP-associated CK2 activity is downregulated in response to DNA damage

CK2 has been implicated in the DNA damage response (Bidwai et al. 1995; Toczyski et al. 1997). Given the requirement for CK2 in pol III transcription in unperturbed cells, a change in the biochemical properties of TBP-associated CK2 could potentially mediate the impaired transcriptional activity of TFIIIB in DNA-damaged cells. This idea was examined by isolating TBP-immune complexes from wild type cells that express GST-tagged CK2 subunits in addition to endogenous CK2. The expression of GST-tagged CK2 subunits was unchanged after UV irradiation (Fig. 4-5, lanes 1,2,6,7,11,12). While there is no change in GST-β or GST-β' recovery following UV irradiation (Fig. 4-5, lanes 3,4,8,9), the recovery of GST-α' in TBP immunecomplexes declines to the baseline level represented by non-specific binding of GST-α' to Protein A Sepharose beads (Fig. 4-5, lanes 13-16). The dissociation of the catalytic subunit from TBP complexes following DNA damage, with a concomitant decrease in the TBP-associated CK2 activity (Fig. 4-5, B, C), provides a plausible molecular mechanism for the DNA damage-dependent repression of pol III transcription.

Changes in the nucleo-cytoplasmic localization of CK2 in response to physiological cues have been documented and may partly confer the substrate specificity of the kinase (Faust and Montenarh 2000). In order to determine if the dissociation of CK2α' from TBP complexes results in changes in its subcellular redistribution, nuclear and cytoplasmic fractions were prepared from strains that express GST-α' or GST-β. As expected, TBP was only recovered in the nuclear fractions (Fig. 4-6). Moreover, the CK2 content of the nuclear fractions remained apparently unchanged after treatment of cells with MMS.

DNA damage-induced transcriptional repression in vivo is mediated via CK2 and a potential CK2 phosphoacceptor site in TBP

To further study the role of CK2 in genotoxic stress signaling to the pol III transcriptional machinery, tRNA and 5S rRNA synthesis in wild type cells and CK2 mutants exposed to UV irradiation was monitored. It was reasoned that if pol III downregulation in response to DNA damage involves CK2, then mutations that perturb CK2 function should dampen damage-induced transcriptional repression.

UV irradiation of 60 J/m^2 results in a 5-fold (means of four independent experiments) decline in tRNA transcription in wild type cells (Fig. 4-7, A, lanes 1 and 2; quantitative results are represented in panel B). At the permissive temperature (27°C) the cka2^{ts} mutant displays reduced CK2 activity in vitro and impaired pol III transcription in vivo (Fig. 2-2, Chapter 2, p 43). UV irradiation of these cells results in1.8-fold repression in tRNA transcription (Fig. 4-7, lanes 3,4). Similar to the cka2^{ts} strain, cells in which CK2β is deleted have a defect in TBP-associated CK2 activity (Fig. 3-5, Chapter 3) and pol III transcription (Fig. 3-6, Chapter 3, p 68). The response of the pol III transcriptional machinery to UV irradiation in CK2β null cells (strain ckb1Δ) is also dampened compared to wild type cells (Fig. 4-7, lanes 5, 6 panel A, quantitation shown in panel B). Collectively these findings demonstrate that properties of CK2 as defined by the cka2^{ts} and ckb1Δ mutations are essential for the enzyme's ability to activate transcription under benign conditions and to respond to DNA damage signals. In support of this idea, cells with mutations in both catalytic and regulatory CK2 subunits (cka2^{ts} ckb1Δ strain) are markedly impaired in pol III transcription in vivo and fail to repress transcription after UV treatment (Fig. 4-7, lanes 7, 8, panel A; quantitation in panel B).

A conserved CK2 consensus site in yeast TBP, Ser128, is likely important for the regulation of pol III transcription by CK2 (Fig. 2-13, Chapter 2, p 54). If Ser128 phosphorylation plays a role in transcriptional regulation in cells with DNA damage, then its substitution by a nonphosphorylatable residue should at least partly block pol III repression in response to DNA damage. Polymerase III transcription is impaired in S128A cells at the permissive temperature and further repressed upon UV irradiation. However, the magnitude of repression of pol III transcription in response to irradiation is 3-fold lower in S128A than wild type cells (Fig. 4-8, lanes 1-4, quantitation in panel

B). In agreement with a previous report, the P65S mutant of TBP is also impaired in pol III transcription (Schultz et al. 1992). But unlike the S128A mutant, it maintains a fold of repression that is similar to that of the wild type cells (Fig. 4-8, lanes 5,6). Therefore the dampening effect of S128A mutation on DNA damage-dependent transcriptional repression is not a generic property of functionally compromised TBP molecules. Significantly, the full transcriptional response in the P65S mutant further indicates that the DNA damage-induced repression of transcription is normally additive to a preexisting defect in transcription. The reduction in the fold of repression in the S128A mutant is therefore consistent with involvement of ser128 in transcriptional repression after DNA damage. Collectively, these results supports a model in which the phosphorylation of TBP by CK2 is a critical target of the genotoxic stress response in yeast.

The in vivo transcription experiments also reveal that the synthesis of pol I and pol III gene products is coordinately downregulated following DNA damage (Fig. 4-7, quantitation of 5.8S rRNA is shown in Fig. 4-9. The poor resolution of the pol I-encoded 25S and 18S ribosomal RNA in this gel system precludes their reliable quantitation). Furthermore, CK2 and TBP S128A mutants display impaired repression of pol I-directed rRNA synthesis in response to DNA damage (Fig. 4-7; Fig 4-8; quantitation in Fig. 4-9). Thus downregulation of TBP phosphorylation by CK2 results in coordinated repression of pol I and pol III transcription after DNA damage.

Discussion

The data presented in this chapter provide new insights into the DNA damage response. It is demonstrated that coordinated downregulation of rRNA and tRNA synthesis is a hallmark of the DNA damage response in yeast. This response is largely governed via the TBP-associated CK2. Although the role of CK2 in mediating transcriptional repression was not previously suspected, CK2 has been implicated in the DNA damage response. Thus ckb2 null yeast cells have a subtle UV sensitivity phenotype (Bidwai et al. 1995). Mutations in either ckb1 or ckb2 also impair adaptation in yeast with an unrepairable double strand break (Toczyski et al. 1997). Since this effect is not due to loss of viability in the arrested mutants, CK2 regulatory subunits likely function in some aspect of overriding the G2/M checkpoint.

Accordingly, deletion of *RAD9*, a central transducer of the checkpoint pathways in yeast, relieves the adaptation-defective phenotype in these mutants (Toczyski et al. 1997).

In mammalian cells, CK2 is recovered in complex with p53 (Keller et al. 2001). CK2 phosphorylates p53 ser 392 in vitro and alanine substitution in this residue reduces p53 transcriptional activity in UV irradiated cells. These data suggest that CK2 may be required for p53-mediated cell cycle arrest and apoptosis (Keller et al. 2001). Further evidence for involvement of CK2 in DNA damage response is provided by the partial complementation of the UV sensitivity of xeroderma pigmentosum (XP) cells by overexpression of CK2β (Teitz et al. 1990). XP cells are primarily defective in the incision step of the nucleotide excision repair process (Drapkin et al. 1994). It may be that the prolonged cell cycle progression in cells that overexpress CK2β provides more time for DNA repair, thereby reducing UV sensitivity (Roussou and Draetta 1994; Li et al. 1999).

Why repress pol III transcription in response to DNA damage?

Transcription-coupled DNA repair results in preferential repair of pol II transcribed genes (Bohr et al. 1985). From a kinetic viewpoint, however, ongoing pol I and pol III transcription confers no advantage due to the lack of gene specificity in repair of pol I and pol III transcribed genes (Aboussekhra and Thoma 1998). In fact, DNA-bound transcription factors are known to be inhibitory to repair (Aboussekhra and Thoma 1998; Conconi et al. 1999). The dissociation of CK2 catalytic subunits after DNA damage likely yields a hypophosphorylated TFIIIB that is impaired in DNA binding (Fig. 2-14, Chapter 2, p 55). The removal of TFIIIB, in turn, precludes transcription initiation by RNA polymerase III which should further allow DNA access by the repair machinery.

The correlation between the rate of cellular protein synthesis and cell cycle progression is well documented; mutations in *S. cerevisiae* translation initiation factor Cdc63 result in a predominantly G1/S arrest (Polymenis and Schmidt 1997). This cell cycle arrest is due to a defect in translation of G1 cyclins mRNA that are disproportionately sensitive to a reduction in cellular protein synthesis capacity (Polymenis, 1997). Moreover, a temperature sensitive mutation in the 53kD subunit

of yeast RNA polymerase III leads to late G1 arrest (Mann et al. 1992). Conversely, overexpression of the normally limting mRNA cap binding protein eIF4E in human fibroblasts leads to cellular transformation (Lazaris-Karatzas et al. 1990). Collectively, these results indicate that progression through cell cycle is in part mediated by the regulated translation of the components of the cell cycle machinery. It follows that the coordinated repression of pol I and pol III transcription may decrease the translational capacity to levels at which key cell cycle regulators are not synthesized in sufficient amounts to promote cell cycle progression. In agreement with this idea, arrest in G1/S and G2/M in yeast with DNA damage is coordinated with reduced translation of G1 cyclins and Cdc28 mRNA, respectively (Azzam et al. 1997; Sidorova and Breeden 1997). In this regard, progression through G1/S and G2/M is blocked in cka2^{ts} cells at the restrictive temperature (Hanna et al. 1995), suggesting that the upregulation of tRNA and rRNA transcription may partly underlie the positive role of CK2 in cellular proliferation in yeast.

TBP-associated CK2 as the effector kinase in a genotoxic stress signaling pathway

The repression of TBP-associated CK2 activity in response to DNA damage is evidently due to dissociation of α/α' from the TBP-CK2 complex. The signaling pathway and the mechanism that mediate this event are unknown. This mode of regulation, however, has a precedent in mammalian cells, where dissociation of CK2α from protein phosphatase 2A (PP2A) underlies the inactivation of PP2A activity in response to platelet-derived growth factor (Hériche et al. 1997).

CK2β is required for maximal kinase activity of the TBP associated-CK2 and as such is required for efficient pol III (and pol I) transcription. Deletion of CK2β alone reduces the fold of transcriptional repression after DNA damage, and in combination with a mutant allele of CK2α', effectively abolishes this response. Since CK2β likely stabilizes the TBP-CK2 complex in unperturbed growth (Chapter 3), targeting of CK2β by checkpoint signaling can, in principle, destabilize this complex. The extensive interactions between CK2α and CK2β is thought to induce conformational changes in the catalytic subunit that underlie the increased kinase activity of the CK2 holoenzyme compared to the catalytic subunits alone (Chantalat et

al. 1999). It is conceivable that binding of a downstream signaling effector to CK2β may induce conformational changes in CK2α that destabilize its binding to TBP. From a mechanistic viewpoint, this is analogous to the activation of protein phosphatase 2B by binding of calmodulin to its regulatory subunit (Coghlan et al. 1995). Whether the effect of CK2β in transcriptional response after DNA damage is via its regulation of the TBP-CK2α/α' complex is not known. However, the absence of transcriptional repression in cka2^{ts} ckb1Δ strain indicates that this response is governed via the TBP-associated CK2 holoenzyme.

It is noteworthy that neither the expression level, nor the nuclear localization of CK2α' is altered in response to DNA damage (Fig. 4-6). This may necessitate a mechanism in order to maintain the dissociation of CK2 α/α' from the TBP complexes until completion of DNA repair. A recent study in HeLa cells indicates that heat shock, and likely UV irradiation, result in specific relocalization of a subset of nuclear CK2α to small foci in the nucleolus (Gerber et al. 2000). This observation suggests that changes in subnuclear compartmentalization of CK2, similar to variations in its nucleocytoplasmic redistribution, may determine its substrate specificity, and by extension its functions, in vivo.

Coregulation of pol I and pol III transcription in cells with DNA damage

The coupling of pol I and pol III transcription is well-documented; yeast with defects in protein secretion exhibit impaired transcription of tRNA and rRNA via activation of a PKC-mediated pathway (Li et al. 2000; Nierras et al. 1999). Similarly, temperature sensitive mutations in the 160kD subunit of RNA polymerase III that reduces tRNA and 5S rRNA synthesis, also inhibits processing of the pol I-encoded 35S rRNA precursor into mature RNA species (Briand et al. 2001). Conversely, a yeast RNA polymerase I mutant displays elevated levels of pre-tRNA (Clarke et al. 1996). The mutual adjustment of pol I and pol III output maintains a steady state ratio of rRNA/tRNA that is remarkably similar to that of the wild type level. An examination of the data (quantitation in Fig. 4-7, 4-8, 4-9) reveals that the fold of repression of pol III-encoded tRNA/5S rRNA and pol I-encoded 5.8 rRNA is similar in UV-irradiated wild type cells. The coordinated downregulation in synthesis of the RNA components involved in protein synthesis ensures efficient utilization of cellular

resources by diverting these resources from the energetically costly process of transcription to DNA repair and other processes required to maintain viability while the damage is repaired (Gasch et al. 2001).

Mutations in CK2α' and CK2β impair in vivo pol I and pol III transcription during unperturbed growth. Furthermore, the fold of downregulation in tRNA and rRNA synthesis is strikingly similar in each UV-irradiated CK2 mutant (compare quantitation in Figs. 4-7 and 4-9). The corresponding levels of pol I and pol III transcription in these mutants, both in normal growth and after genotoxic stress, is suggestive of constitutive mechanical coupling of these processes via TBP associated-CK2. In support of this idea, mutation in TBP Ser 128, the putative CK2 phosphorylation site in TBP, also results in comparable downregulation of pol I and pol III transcription (compare quantitation in Figs. 4-8 and 4-9). Collectively, these observations are consistent with the idea that regulation of TBP phosphorylation by CK2 underlies coordinated expression of tRNA and rRNA.

TFIIIB is a key target of diverse regulatory mechanisms that determine the output of the pol III transcribed genes in response to different physiological cues (White et al. 1995; Kassavetis et al. 2001). The repression of transcription in yeast with DNA damage is also mediated by signaling events that impinge on TFIIIB. This response occurs via a subset of cellular CK2 that is associated with TFIIIB. CK2 and TFIIIB are conserved in eukaryotes, as are many components of the DNA damage checkpoints. These observations raise the possibility that CK2 mediates DNA damage signaling to the pol III transcription machinery in all eukaryotes. Preliminary support for this idea is indicated by the inhibition of small RNA synthesis, that are not transcribed by pol II, in UV irradiated HeLa cells (Eliceiri 1979).

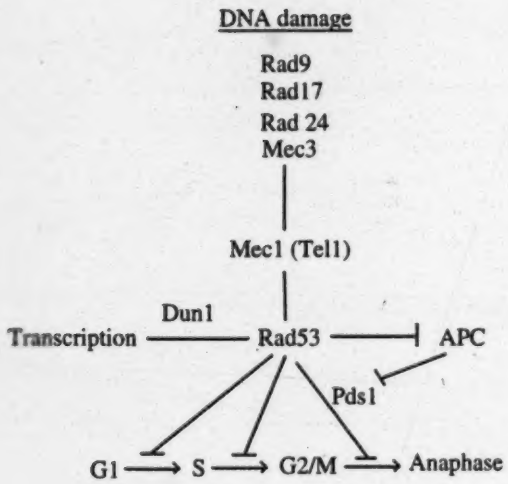

Figure 4-1. A current view of the genetic organization of the checkpoint pathways in *S. cerevisiae*. The Rad9, Rad17, Rad 24, and Mec3 act at an early stage of checkpoint activation as sensors of DNA lesions or signal modifiers. Two essential genes, *MEC1* (*TEL1*) and *RAD53* form the central conduit for checkpoint signal transduction. Cell cycle arrest and reduction in the rate of DNA replication require these two genes. Dun1 kinase activity is increased in cells with DNA damage in a Mec1/Rad53-dependent manner and this activation is required for transcriptional activation of the genes encoding ribonucleotide reductase. G1/S and intra-S phase arrest is partly mediated via inhibition of cyclin-dependent kinases. In normal growth, progression through mitosis requires degradation of Pds1 by ubiquitin-dependent proteolysis mediated by the anaphase promoting complex (APC). Inhibition of APC by Rad53 results in stabilization of Pds1 and blocking progression through M phase (see text for details).

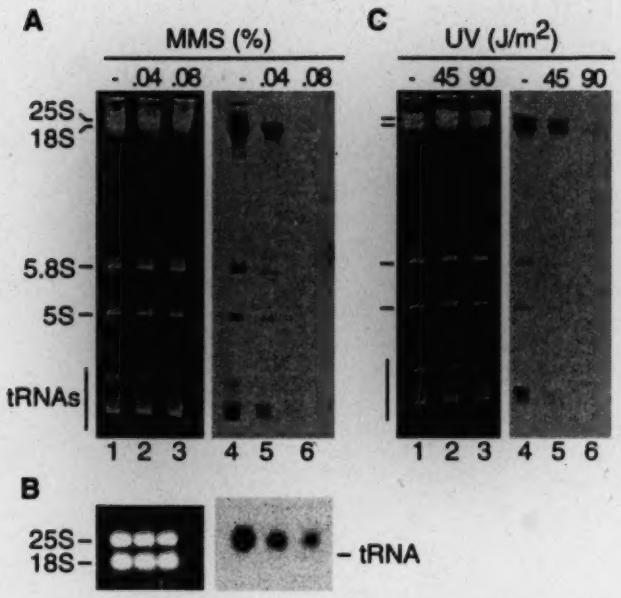

Figure 4-2. Genotoxic stress represses RNA polymerase III transcription in vivo. A. Analysis of transcription in MMS treated cells by metabolic labeling. ^{3}H-uracil was added to CKA2 cultures after 90 min of growth in the presence of MMS as indicated. After 30 min of labeling, total RNA samples were prepared and 10μg aliquots resolved by denaturing PAGE. The gel was stained with ethidium bromide, photographed (left panel), then processed for fluorography (right panel). **B.** S1 nuclease protection analysis of $tRNA^{Trp}$ transcription in cells treated with MMS. RNA was isolated from CKA2 cells treated for 2 hr with MMS as in **A**. Transcripts in 10 μg samples were hybridized to an end-labeled oligonucleotide probe, digested with S1 nuclease, and the products resolved by denaturing PAGE (right panel). Equivalent total RNA recovery was confirmed by electrophoresing 5μg of each starting sample in a 1% formaldehyde-agarose gel and staining with ethidium bromide to detect the large rRNAs (left panel). **C.** Analysis of transcription in UV irradiated cells by metabolic labeling. CKA2 cells were exposed to the indicated doses of UV irradiation and grown for 1 hr prior to labeling for 30 min with ^{3}H-uracil. Panels as in **A**.

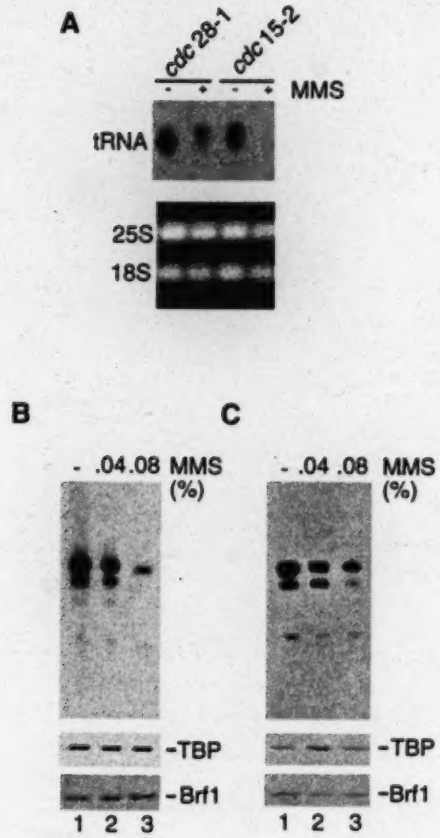

Figure 4-3. Pol III transcription is repressed in extracts from cells with DNA damage. **A.** Analysis of $tRNA^{Trp}$ transcription in MMS treated *cdc* mutants by an S1 nuclease assay. Cells were heat shocked for 2 hours at 37°C to induce cell cycle arrest. They were then treated for 2 hours with 0.08% MMS at 37°C before isolation of RNA (panels as in Fig. 4-1). **B.** Pol III transcription is repressed in whole cell extracts from MMS-treated cells. Run-off transcription directed by a $tRNA^{Tyr}$ gene was assayed in 10μg of extract from cells treated for 2 hours with MMS (top panel). TBP and Brf1 recovery in equal μg of each extract was monitored by immunoblotting (lower panel). **C.** MMS-dependent repression of pol III transcription is preserved in a pol III-enriched fraction. Whole cell extracts prepared after MMS treatment were chromatographed in parallel on DEAE-Sepharose to obtain a 0.3 M KCl fraction that contains all components of the pol III transcriptional machinery. Transcription (in 4μg aliquots) and TBP/Brf1p recovery were assayed as in **B**.

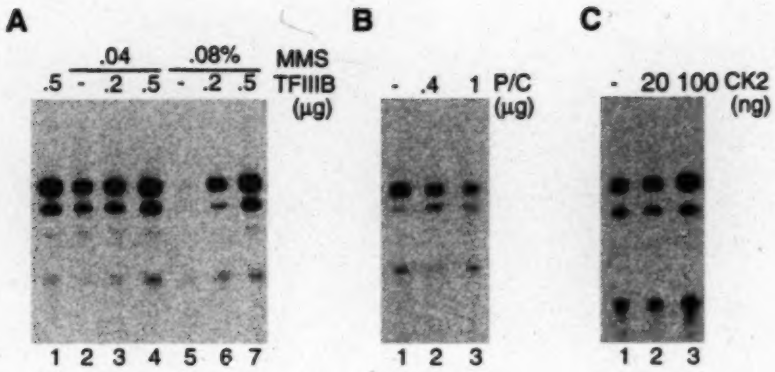

Figure 4-4. TFIIIB restores transcription in extracts from DNA-damaged cells. A. TFIIIB from control cells was added in the indicated amounts to whole cell extracts from untreated (lane 1) or MMS-treated cells. $tRNA^{Tyr}$ transcription was monitored as in Fig. 4-2. **B.** An active pol III/TFIIIC fraction (P/C) does not restore the transcriptional activity of MMS-treated extract. The transcriptionally active P/C fraction was titrated into extract from cells treated with 0.08% MMS. **C.** CK2 stimulates pol III transcription in extract from MMS treated cells. CK2 purified from wild type untreated cells was added to extract from cells treated with 0.08% MMS. The film exposures in **B** and **C** were prolonged to clearly demonstrate the effects of added fractions.

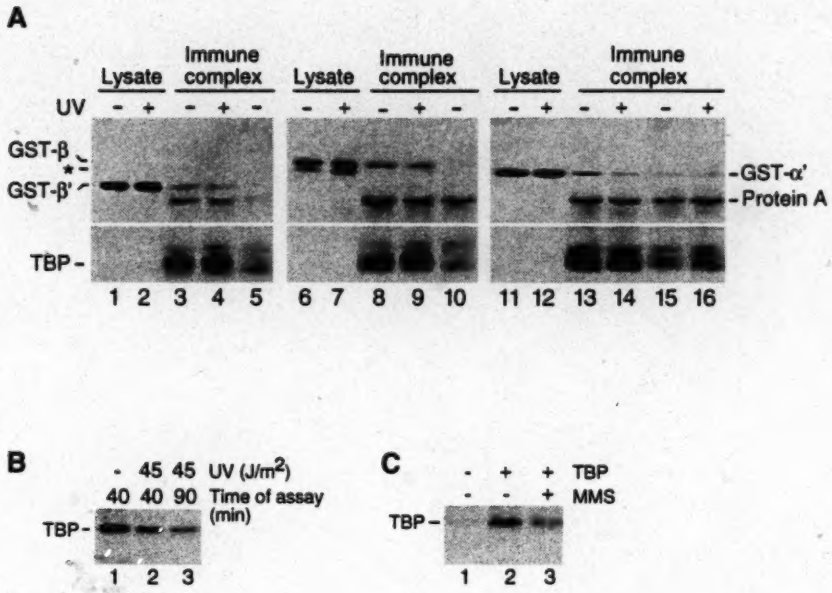

Figure 4-5. Regulation of TBP-associated CK2 in cells exposed to genotoxic stressors. A. The subunit composition of TBP-associated CK2 in UV irradiated cells expressing GST-β' (lanes 1-5), GST-β (lanes 6-10), or GST-α' (lanes 11-16). Lysates were prepared after UV irradiation (60 J/m^2) and 4 μg of each lysate was monitored for expression of GST-CK2 fusion constructs by immunoblotting against GST. TBP complexes were recovered by immunoprecipitation using an anti-TBP antibody (lanes 3, 4, 8, 9, 13, 14) or preimmune serum (lanes 5, 10, 15, 16), and probed with anti-GST (upper panels) or anti-TBP (lower panels) antibody. The smear comigrating with TBP in lanes 5, 10, 15, 16 is the IgG light chain. **B.** TBP-associated kinase activity is downregulated in UV irradiated cells. Kinase activity was measured in TBP immune complexes from untreated and UV-treated cells. TBP immune complexes were prepared 40 and 90 min after UV irradiation and assayed as in Fig. 3-4 using 120μg/ ml recombinant TBP as substrate. **C.** TBP-associated CK2 activity is downregulated in MMS-treated cells. Kinase activity was measured as above in TBP immune complexes from cells untreated or treated with 0.08% MMS for 2 hrs.

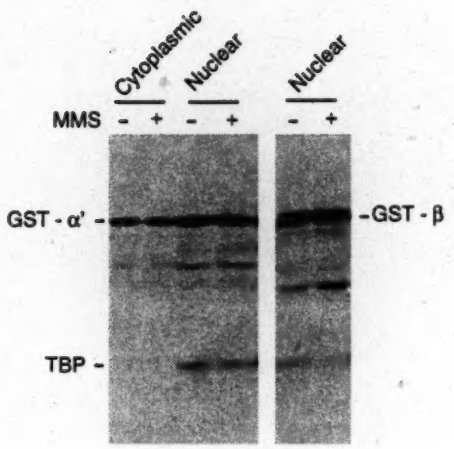

Figure 4-6. Subcellular localization of CK2 subunits in cells grown under benign conditions and after genotoxic stress. Cells that harbor GST-tagged CK2α' or CK2β were treated with 0.08% MMS. After 90 min, nuclear and cytoplasmic fractions were prepared and subsequently monitored for CK2 and TBP content by Western blotting. Note the exclusive localization of TBP to the nuclei.

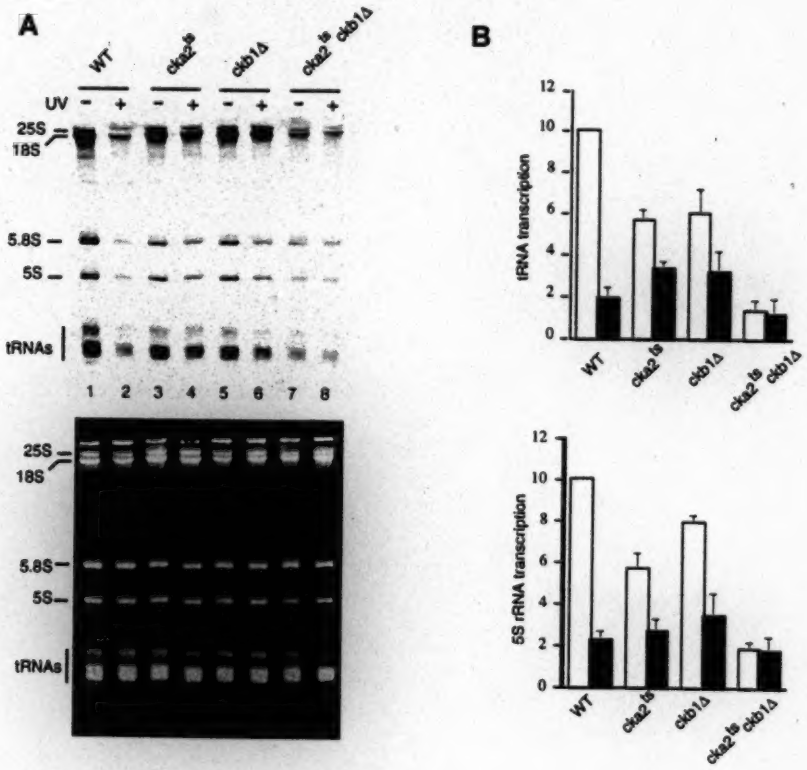

Figure 4-7. Full transcriptional repression in response to DNA damage requires active CK2.
A. A temperature sensitive strain of CK2 ($cka2^{ts}$), a CK2β null mutant (ckb1Δ), the $cka2^{ts}$ ckb1Δ double mutant, and their parental wild type (WT) grown at 27°C were UV irradiated (60 J/m^2) and grown for an additional 1 hr prior to labeling for 30 min with ^{3}H-uracil. Panels as in Fig. 4-1. **B.** Quantitation of pol III transcription of the tRNA and 5S rRNA genes in untreated (open bars) and UV irradiated (filled bars) CK2 mutants. The graphs plots transcription in arbitrary units with wild type normalized to 10, and show the means and the standard deviation of four independent experiments.

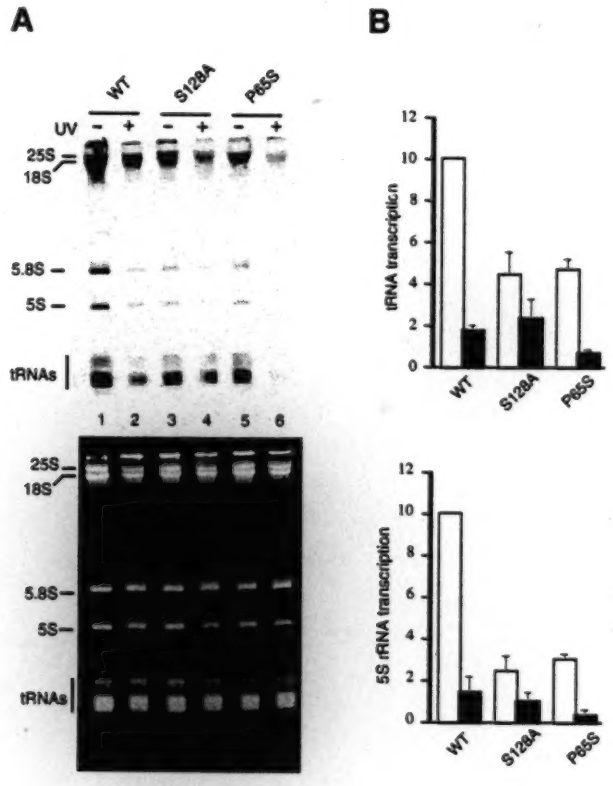

Figure 4-8. Full transcriptional repression in response to DNA damage requires intact S128 of TBP. A. Transcriptional response of untreated and UV treated wild type (WT) and TBP mutants (S128A and P65S) grown at 27°C. Metabolic labeling was performed as in Fig. 4-6. **B.** Quantitation of DNA damage effects on pol III transcription in TBP mutants. Panels as in Fig. 4-6.

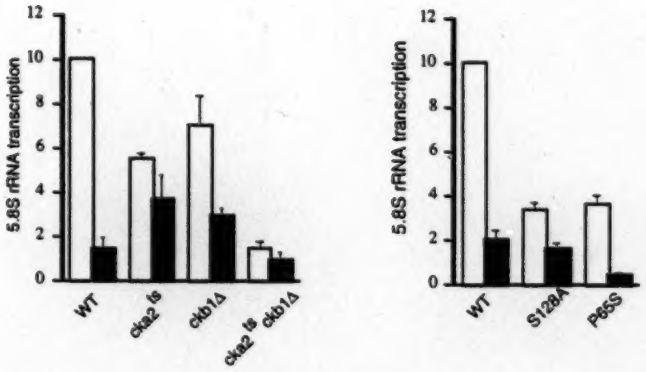

Figure 4-9. CK2 and TBP S128 are required for efficient pol I transcription under benign conditions and mediate pol I transcription after DNA damage. Quantitation of the pol I transcribed 5.8S rRNA in untreated (open bars) and UV treated (filled bars) CK2 (left panel) and TBP mutants (right panel). In vivo labelled RNA, as depicted in Fig. 4-6, **A**, and Fig. 4-7, **A**, were used to quantitate 5.8S rRNA.

Chapter 5

Perspectives and future experiments

Summary of the regulation of pol III transcription by CK2

Using a combined genetic and biochemical approach in *S. cerevisiae*, it is shown here that a subset of cellular CK2 is both physically and functionally associated with the pol III transcription factor TFIIIB. CK2 phosphorylates and activates TFIIIB DNA binding and in doing so, promotes pol III transcriptional initiation. This effect is likely mediated by direct phosphorylation of the TBP subunit by CK2 (Fig. 5-1).

The regulation of TBP by CK2 evidently constitutes the mechanism that mediates the repression of pol III transcription after DNA damage. Thus the targeted dissociation of the CK2 catalytic subunits from TBP is expected to yield a hypophosphorylated TFIIIB that is impaired in DNA binding. The resultant reduction in pol III transcription may allow for repair of the pol III transcribed genes, facilitate the induction of cell cycle arrest, and enhance cellular viability by diverting metabolic resources to DNA repair and other processes required for maintaining homeostasis in cells with DNA damage. It is speculated that reconstitution of TBP-CK2 holoenzyme complex following the completion of DNA damage, restores pol III transcription.

Future studies in the regulation of pol I and pol III transcription by CK2

Biochemical characterization of pol I repression after DNA damage

In unperturbed growth, $cka2^{ts}$ and ckb1Δ strains are impaired in pol I and pol III transcription, as is the TBP S128A mutant. This observation suggests that CK2 phosphorylation of TBP, a core transcription factor shared between the pol I and pol III transcription machineries, allows for the coordinated expression of tRNA and rRNA. This regulatory mechanism evidently controls the output of pol I and pol III transcription in response to DNA damage, further suggesting that the role of CK2 in pol I transcription may be mediated by its direct association with TBP. A scheme for fractionation of yeast SL1, the pol I TBP-containing core transcription factor, has been described (Clarke et al. 1996). Probing this fraction for CK2 would verify if a subset of cellular CK2 is functionally dedicated to pol I transcription. Furthermore, the in vivo downregulation of pol I transcription in cells with DNA damage can be examined

in vitro by using S1 nuclease protection assay to monitor the capacity of extracts from UV irradiated cells in directing transcription from a pol I promoter (Zaragoza et al. 1998). This biochemical approach can be subsequently used in a conventional add back experiment to examine if SL1 is specifically targeted by DNA damage signaling.

It was postulated that coordinated repression of tRNA and rRNA expression in response to DNA damage results in reduced protein synthesis in vivo (Chapter 4). The reduced rate of translation, in turn, may partly underlie the checkpoint activated cell cycle arrest. Accordingly, cka2^{ts} cells grown at the permissive temperature (25^{o}C) are impaired in pol I and pol III transcription and display a nearly three fold reduction in protein translation (Hanna et al. 1995). However, the observation that CK2 phosphorylation regulates the in vitro activity of translation elongation factor EF-1α (Sheu and traugh 1999) necessitates establishing a direct correlation between the reduced pol I and pol III transcription and impaired cellular protein synthesis. Preparation of a yeast extract that supports translation has been described (Zinser and Daum 1995). Examining the rate of protein synthesis from an exogenous RNA template in extracts from UV irradiated wild type cells will reveal if the observed repression of transcription is coordinated with a reduction in protein synthesis capacity.

Identification of signaling pathways that mediate the repression of transcription after DNA damage

Inactivating mutations in checkpoint genes result in increased cell death by relieving the dependency of the cell cycle progression on completion of DNA replication or repair. Accordingly, loss of viability has traditionally provided a marker for conducting genetic screens in order to identify genes that function in checkpoint pathways. However, since these screens rely on differential sensitivity of mutants to DNA damaging agents, they have often yielded genes that have no known functions in checkpoint signaling and are instead required for DNA repair or maintaining cellular homeostasis in cells with DNA damage (Paulovich and Hartwell 1995; Weinert 1998; Birrell et al. 2001). Unlike loss of viability, the repression of transcription after DNA damage is an active and likely direct cellular response. Therefore, it can in principle provide a novel biochemical marker for identification of genes that function as sensors

of DNA lesions, signal transducers, or downstream effectors that execute transcriptional repression.

A collection of over 4600 *S. cerevisiae* strains that harbor individual disruptions of non-essential ORFs is commercially available (Research Genetics, Huntsville, AL). Systematic analysis of in vivo ^{3}H-labelled tRNA and rRNA in these mutants in response to MMS treatment could identify genes that when disrupted, dampen or block repression of transcription after DNA damage. The utility of this approach is demonstrated by the identification of CK2 and TBP as the downstream mediators of the DNA damage-induced transcriptional repression (Chapter 4). Conducting this screen requires gel electrophoretic analysis of RNA isolated from a 2 ml yeast culture and is therefore amenable to simultaneous screening of 40-50 strains. Since this screen is not high throughput, it is primarily intended for initial identification of a gene which can be subsequently exploited, in suppression screens and coimmunoprecipitation assays, to identify other checkpoint factors. The identification of these components should prove informative in elucidating the mechanism that underlies dissociation of the CK2 catalytic subunits from TBP in cells with DNA damage. Importantly, since checkpoint pathways often share upstream components, this approach may yield novel genes that mediate other cellular responses, such as cell cycle arrest and transcriptional induction of genes involved in DNA repair, in addition to repression of transcription.

Cellular transcriptional repression after DNA damage and in response to other forms of stress

MMS and UV irradiation generate diverse DNA lesions and have therefore been extensively used in studying cellular responses to DNA damage. However, these genotoxic agents also modify cellular proteins, lipids, and RNA (Boffa and Bolognesi 1985; Bolognesi et al. 1988; Rosette and Karin 1996). This raises the possibility that the observed changes in tRNA and rRNA expression after MMS and UV treatment may be due to cellular modifications other than DNA damage.

It is known that UV irradiation of enucleated mammalian cells results in activation of the environmental stress response (ESR), a signaling pathway that is thought to be involved in maintaining cellular homeostasis in response to general

environmental perturbations such as heat shock and amino acid starvation (Devary et al. 1991; Devary et al. 1993). Importantly, DNA microarray analysis in yeast reveals that changes in cellular mRNA expression following heat shock or MMS treatment are strikingly similar (Gasch et al. 2000; Gasch et al. 2001). However, a functional Mec1 pathway is required for expression of the ESR genes after treatment with MMS despite the fact that variation in cellular mRNA transcription after heat shock is Mec1-independent (Gasch et al. 2001). Since Mec1 is a central component of several DNA damage checkpoint pathways, these observations suggest that activation of ESR may be an integral part of the overall cellular response to DNA damage. This idea can be examined in yeast strains in which inducible expression of nucleases generate DNA lesions within predetermined genomic loci (Lee et al. 1998; Mills et al. 1999). Monitoring tRNA and rRNA expression in these cells following the induction of double strand breaks should determine if the repression of transcription is a DNA damage-specific cellular response.

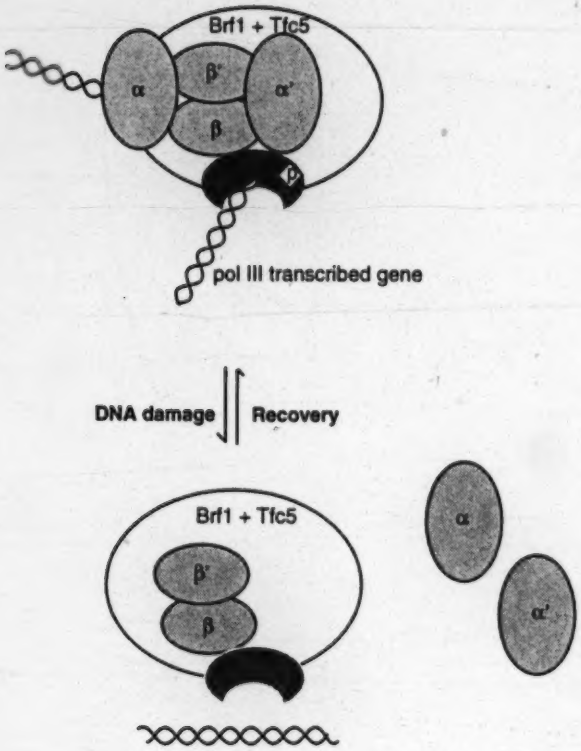

Figure 5-1. A model for the regulation of pol III transcription by CK2. In normal growth, CK2 is associated with TFIIIB. This interaction is mediated in part by direct binding of CK2β subunit to TBP. CK2 may directly phosphorylate TBP on Ser 128. This results in increased recruitment of TFIIIB to the upstream region of a pol III transcribed gene, and enhanced pol III transcription. In cells with DNA damage, dissociation of CK2 catalytic subunits may yield a hypophosphorylated TBP. This results in impaired DNA binding of TFIIIB and reduced pol III transcription. It is postulated that re-association of CK2 catalytic subunits with TFIIIB restores pol III transcription after completion of DNA repair. The putative signaling pathways and the mechanism by which they mediate the dissociation of CK2 catalytic subunits are uncharacterized.

Chapter 6

Experimental methods

Buffers

The buffers were: YDBI: 20mM Hepes-KOH (pH 7.9), 50 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 0.4 mM PMSF, 0.3 μg/ml leupeptin, and 20% glycerol; buffer B: 20mM Hepes-KOH (pH 7.9), 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 0.3 μg/ml leupeptin, and 20% glycerol; buffer L: 20mM Hepes-KOH (pH 7.9), 1 mM EDTA, 5 mM $MgCl_2$, 2.5 mM DTT, 0.2 mM PMSF, 10% glycerol, and 0.05% NP-40; buffer K: 20mM Hepes-KOH (pH 7.9), 0.5 mM DTT, 0.4 mM PMSF, 0.6 μg/ml leupeptin, 0.6 μg/ml pepstatin, and 20% glycerol; buffer T: 20 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM DTT, 1mM PMSF, 2 mM benzamidine-HCl, and 10% glycerol. Unless stated otherwise, numbers following the buffers' name indicate salt concentration in mM, e.g., B80 denotes buffer B that contains 80 mM salt.

Construction of yeast mutants

The CKB1 gene, encoding CK2β, was disrupted in isogenic CKA2 and $cka2^{ts}$ strains (YDH6 and YDH8, described in Hanna et al. 1995) to construct ckb1Δ and $cka2^{ts}$ ckb1Δ, respectively. An integrating plasmid was obtained by PCR using oligonucleotides 5'CCGAATCTGTAAATTGGTGAATTGGTATTCCGAATCTGTAAATTGGTGAATTGGTATTTAGTTTAGAAGCGACCATTAAGAGAGAGAAAACTCGTACGCTGGTCAGand5'GGTAAAGTACATATGTTGGTAGGCGATGAATTCGAGCTG (underlined residues are complementary to the region immediately flanking the *CKB2* open reading frame, and the residues at the 3' ends are the sequence complementary to the region flanking the *kan*^{r} gene). Using these oligonucleotides, the *kan*^{r} gene was PCR amplified from the *kan* MX4 module (Wach et al. 1994) and transformed into yeast cells which were subsequently selected on plates containing 200 μg/ml geneticin (G418; Sigma Chemicals). The selected transformants were initially assayed by PCR and subsequently probed by Southern blotting, using end-labeled Sal1-Sca1 fragment of the *kan*^{r} gene, to confirm proper integration.

TBP Ser128 was substituted in plasmid pSH225 using the oligonucleotide 5'-GCTTTAATTTTTGCGTCGGAAAATGGTTGTTACCGGTGCAAAAAGTGAGGATGACTC and the Transformer Site-directed Mutagenesis Kit (Clontech, CA). The

underlined residues were changed to GCT or GAT to replace Ser128 with Ala (A) or Asp (D), respectively. Plasmid-borne wild type TBP in an *spt15Δ* background was replaced by plasmid shuffling with pSH225 containing wild type, S128A, or S128D TBP (Schultz et al. 1992; reagents kindly provided by S. Hahn).

Preparation of transcription extract

Yeast S100 whole cell transcription extracts, from cells grown to A_{600} of 0.5-1, were prepared as described (Hockman and Schultz 1996). Cells were broken under liquid nitrogen in a pestle and mortar. A motorized mortar grinder was used for large scale extract preparation. Nuclear extracts were prepared according to Dunn and Wobbe (1995); spheroplasts were obtained by treating yeast cells with zymolyase 100T (Seikagaku Chemicals, Japan). To allow metabolic recovery after zymolyase treatment, the spheroplasts were incubated in YPD+1M sorbitol on a rotary shaker. After 1 hour, the spheroplasts were spun down and lysed by resuspending in 20 vol ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 2mM EDTA, 1mM DTT, and 18% (W/V) Ficoll-400 (Amersham Biosciences). After a 10 min spin at 20,000g, the nuclear pellet was recovered and lysed in an equal volume of buffer B100 by using a Dounce homogenizer.

Purification and assay of transcription factors from yeast

pol III transcription factors were fractionated according to Kassavetis et al. (1995), with modifications as noted below (fractionation scheme is depicted in Fig. 6-1). The transcriptionally active 35-70% ammonium sulfate cut (2.8 g total protein) of whole cell extract was dialyzed to YDBI, and applied to 140 ml Bio-Rex 70 column (Bio-Rad). This column was step-eluted with 100, 250 and 500 mM KCl in YDBI. The 500 mM KCl fraction contained all components of the pol III transcription machinery, although it was transcriptionally inactive due to an inhibitor that is separated from pol III/TFIIIC on DEAE and from TFIIIB on hydroxylapatite. The Bio-Rex 500 mM KCl fraction (160 mg) was dialyzed to B100 and applied to a 40 ml DEAE Sepharose Fast Flow (Amersham Biosciences) column equilibrated in B100; fractions were collected from the 100, 300, and 600 mM KCl washes. TFIIIB (108 mg), eluted in the flow-through, was dialyzed to buffer K25. 50 mg of this fraction

was loaded onto a 20 ml Bio-Gel hydroxylapatite (Bio-Rad) column equilibrated in buffer K25. The column was washed with buffer K50 and developed with a 75 ml gradient (50-300 mM KPO_4) which eluted TFIIIB at approximately 200 mM KPO_4. TFIIIB fractions (~3 mg) were dialyzed against buffer B200 and applied to a 3 ml Cibacron Blue-Sepharose column (Fluka Biochemicals). Following washing with buffer B200, TFIIIB was eluted with B1000. Pol III and TFIIIC (20 mg) eluted together in the 300 mM cut from DEAE. 10 mg of this fraction (P/C) was dialyzed to buffer L100, pre-cleared with 0.6 mg pGEM3, and applied to a 1.1 ml TFIIIC affinity column (synthetic Box B+ 29-mer coupled in 10 repeat fragments [average] to Sepharose CL2B (Amersham Biosciences); Kassavetis et al. 1989). The column was washed with buffer L200 and TFIIIC was eluted with L1000. Bulk pol III activity was measured according to Schultz and Hall (1976). TFIIIB was monitored during chromatography by immunoblotting using a polyclonal antiserum against recombinant yeast TBP. TFIIIB was also monitored by its ability to complement fraction P/C, which is transcriptionally inactive and devoid of TBP, but is enriched in bulk pol III activity and TFIIIC DNA-binding activity. TFIIIC was monitored by its DNA binding activity according to the method of Kassavetis et al. (1989). The pol III machinery was obtained in a single fraction (D300) by applying whole cell extract in YDBI to a DEAE Sepharose Fast Flow column (Riggs and Nomura 1990). The column was washed with 90 mM KCl/YDBI and the transcription machinery was then eluted with 300 mM KCl/YDBI.

Expression and purification of recombinant TBP

Yeast TBP was expressed in *E. coli* strain BL21(DE3) and purified according to a protocol described in Reddy and Hahn (1991); cells containing plasmid pSH228 were induced with 0.4 mM IPTG for 2 hr. The soluble fraction resulting from sonication of cells in buffer T50 was applied to DEAE Sepharose Fast Flow (Amersham Biosciences) and the flow-through collected. This material was applied to Mono S (Amersham Biosciences) and eluted with a 50-500 mM KCl gradient in buffer T. TBP was recovered in the 250-300 mM KCl fractions.

Fractionation and in vitro assay of yeast CK2

CK2 was fractionated according to Bidwai et al. (1994) from cells broken under liquid nitrogen using a motorized mortar grinder (purification scheme is depicted in Fig. 6-2). Equal volume of each fraction was assayed for CK2 content by determining phosphorylation of a CK2 substrate peptide as described (Hockman and Schultz 1996), except that a TCA precipitation step was introduced in order to remove polypeptides from the reaction mix prior to spotting onto P81 paper. This step reduces the variability between replicate assays. Specifically, 25 µl reactions were stopped by adding TCA to 5%, incubated on ice for 10 min, and then spun for 5 min in a microcentrifuge. 20 µl of the supernatant was then spotted onto P81 paper and processed for scintillation counting.

In vitro kinase and phosphatase reactions

Fractionated CK2 was incubated with substrates in 20 µl final volume containing 15mM Hepes-KOH (pH 7.9), 130 mM KCl, 10 mM $MgCl_2$, 2.5 mM EGTA, 0.2 mM EDTA, and 1 µl γ^{32}P-GTP or γ^{32}P-ATP (NEN; 3000 Ci/mmol). The reactions were performed for 15 min at 30°C, stopped with SDS sample buffer, and the products resolved by SDS-PAGE. Hydroxylapatite TFIIIB was dephosphorylated by incubation for 20 min at room temperature with the calf alkaline intestinal phosphatase (Sigma Chemicals) in a buffer containing 20 mM Tris-HCl (pH 8.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1mM DTT. Sodium vanadate (Sigma Chemicals) was used at 0.5 mM.

In vitro transcription reactions

Multiple round transcription reactions were performed according to Hockman and Schultz (1996) using pY5S for 5S rRNA transcription (Schultz et al. 1992) and pTZ1, which contains the *SUP4* $tRNA^{Tyr}$ gene, for tRNA transcription (Kassavetis et al. 1989). Details are noted in the figure legends. Transcriptional complementation of $cka2^{ts}$ extract with CK2 and TBP was performed without pre-incubation of CK2 and TBP, since CK2 phosphorylation of TBP prior to their addition to cell extract usually dampens the stimulation of transcription. It may be that in a kinase reaction containing only purified TBP and CK2, TBP is aberrantly phosphorylated on residues that are not normally available when TBP is assembled into TFIIIB.

In vivo transcription

Total RNA was prepared by hot phenol extraction according to Schmitt et al. (1990) from cells grown to early log (A_{600} of 0.2- 0.3). Newly synthesized $tRNA^{Tyr}$ was measured by S1 protection analysis (Hockman and Schultz 1996). Transcription was also monitored by in vivo labeling of 5 ml cultures with [5,6-^{3}H]-uracil (38.5 Ci/mmol, NEN, added to 15 μCi/ml). After 30 min, total RNA was isolated and resolved by electrophoresis in an 8% acrylamide gel containing 7M urea. The gel was stained with ethidium bromide to assess steady state tRNA and rRNA levels, then processed for fluorography (EN^{3}HANCE, NEN). Quantitation of images of stained gels and fluorographs, acquired with a flatbed scanner, was performed using the Image Gauge program (v. 3.0, Fuji). Transcription signals were normalized against RNA recovery as determined by analysis of stained gels.

Electrophoretic mobility shift assay

Assays were performed essentially according to Braun et al. (1989) using 0.5 ng of an end-labeled EcoRI/HinDIII fragment that contains the entire coding region of the *SUP4* $tRNA^{Tyr}$ gene (from pTZ1, kindly provided by Peter Geiduschek). To examine the effect of phosphorylation on DNA binding, TFIIIB was incubated with Potato Acid Phosphatase (PAP, Sigma Chemicals) for 1 hr at 30°C, followed by addition of 30 mM β-glycerophosphate (Sigma Chemicals) to inhibit the phosphatase. As a control, PAP was briefly incubated with inhibitor before adding to TFIIIB. The entire mixture was then added to preformed TFIIIC-DNA complex. After a further 30 min incubation at room temperature, heparin was added (150 μg/ml final concentration) to strip TFIIIC from the DNA. The final composition of all reactions (12μl) was: 20 mM Hepes pH 7.9, 120 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 0.4 mM DTT, 0.3 mg/ml BSA, and 6 μg/ml of pGEM3 as non-specific competitor.

Immunoprecipitation and immune complex kinase assay

Strains expressing GST or GST fusion constructs in plasmid pYEX 4T-1 have been described (Martzen et al. 1999; from Research Genetics, AL). Initially, it was

determined that plasmid-borne GST-β and GST-α' complement the growth defects of ckb1Δ and cka2^{ts} cells, respectively. All steps were performed at 4°C except as indicated. Typically 100 µl of packed early log cells (grown in CM-Ura at room temperature) was resuspended in 1.5 vol of buffer N-150 (30 mM Hepes pH 7.9, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.01% NP-40) and lysed by 20 sec agitation with glass beads in a bead-beater (Biospec, OK). After a 10 minute incubation with DNase I (0.1 mg/ml) and RNase A (50 µg/ml), the lysate was microcentrifuged for 30 minutes to remove cellular debris. 10 µl of packed Protein A-Sepharose beads (Sigma Chemicals) was added to the supernatant for 30 min in a pre-clearing step, and the beads then removed. To immunoprecipitate TBP-containing complexes, rabbit polyclonal antiserum raised against recombinant yeast TBP was then added to 120 µg of supernatant in a volume that gave a final 1:100 (v/v) dilution of the serum. After mixing for 1 hr, 10 µl Protein A-Sepharose beads was added and the reaction incubated for an additional 30 min. The beads were then spun down and extensively washed with buffer N-150 containing 0.1% NP-40. Immune complex kinase reactions were performed at room temperature for 15 min. The final reactions (10 µl) contained: 25 mM Tris pH 8, 12 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, and 1µl [γ-^{32}P]GTP (6000 Ci/mmol, NEN). The entire reactions were boiled in SDS sample buffer, and the products resolved by SDS-PAGE. After electroblotting onto nitrocellulose, the phosphorylated proteins were detected by autoradiography. The membrane was subsequently probed by immunoblotting (Ghavidel and Schultz 1997) to verify equal loading of TBP or to detect GST-tagged proteins.

In vitro assay of TBP binding to purified CK2 subunits

40 µg (400 µl) of recombinant TBP was microconcentrated to 60 µl in an Ultrafree-MC Centrifugal Filter (Millipore) and incubated with 40 µl of CNBr-activated, pre-swelled Sepharose 4B beads (Amersham Biosciences). After overnight incubation at 4°C, the beads were quenched overnight with 1M ethanolamine pH 8, followed by several washes in buffer X-150 (20 mM Tris pH 8, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.01% NP-40). Coupling efficiency was monitored by immnuoblotting against TBP. As a control 40 µg of BSA (New England Biolabs) was similarly cross-linked to beads. The individual regulatory

subunits of CK2 were obtained by gel purification (Cho et al. 1999). Briefly, 120 µg of highly enriched CK2 (heparin agarose fraction; Ghavidel and Schultz 1997) was autophosphorylated, resolved by preparative SDS-PAGE, and the ^{32}P-labeled regulatory subunits detected by wet gel autoradiography. Proteins were eluted from gel slices, denatured in 6M guanidine-HCl, renatured at room temperature, and acetone precipitated. Approximately half of each recovered protein sample was diluted in 20 µl of buffer (X-150 plus 50 µg/ml BSA) and incubated for 90 min at 4°C with 5 µl of Sepharose beads cross linked to TBP or BSA. The beads were then spun down, washed several times in buffer X-150, boiled in SDS-PAGE sample buffer, and electrophoresed.

Antibodies and recombinant proteins

New Zealand white rabbits were immunized and boosted with near homogeneous recombinant yeast TBP prepared as described above. Anti-TBP IgGs were purified by ammonium sulfate precipitation and TBP-Affigel 10 (BioRad) affinity chromatography. Goat anti-GST antibody was from Amersham Biosciences. Anti-Brf1 antibody from Ian Willis was used as described (Sethy et al. 1995). Purified recombinant TBP, Brf1 (from Steve Hahn and I. Willis), and GST-β' were used as standards for quantitative immunoblotting in which chemiluminescence (ECL, Amersham Biosciences) was directly quantitated using a FluorChem digital imaging system and the AlphaEaseFC program (Alpha Innotech Corp.). For expression of yeast CK2β' in E. coli the *CKB2* gene was cloned between the BamHI and EcoRI sites of pGEX-4T-1; the resultant amino-terminally tagged GST fusion protein was batch purified using glutathione sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions.

Immunoblotting

Proteins were resolved on 12 or 15% SDS-polyacrylamide gels (Laemmli 1979), and electroblotted to nitrocellulose membrane in Tris-glycine buffer (Bjerrum et al., 1984) using a semi-dry blotter (Tyler Instruments, Edmonton, AB). The membranes were incubated with antiserum in 20% horse serum in TBST. The immunoreactive species were detected using horseradish peroxidase conjugated anti-

rabbit antibody and the ECL chemiluminescence detection system (Amersham Biosciences).

Induction of DNA damage and assessment of viability after genotoxic stress

30 ml cultures were grown to an A_{600} of 0.2 in CM medium and irradiated with a 254 nm germicidal (UVGL-58) lamp at the dose rate of 1 J/m^2/sec while stirring in a 15 cm Petri dish (Siede et al. 1993). Following UV or MMS treatment, cells were plated and viability was measured by counting the number of colonies after 3 days at room temperature. Under these conditions viability was 52% and 18% after 45 and 90 J/m^2 of UV irradiation, and 70% and 22% after 2 hrs of treatment with 0.04% and 0.08% MMS, respectively.

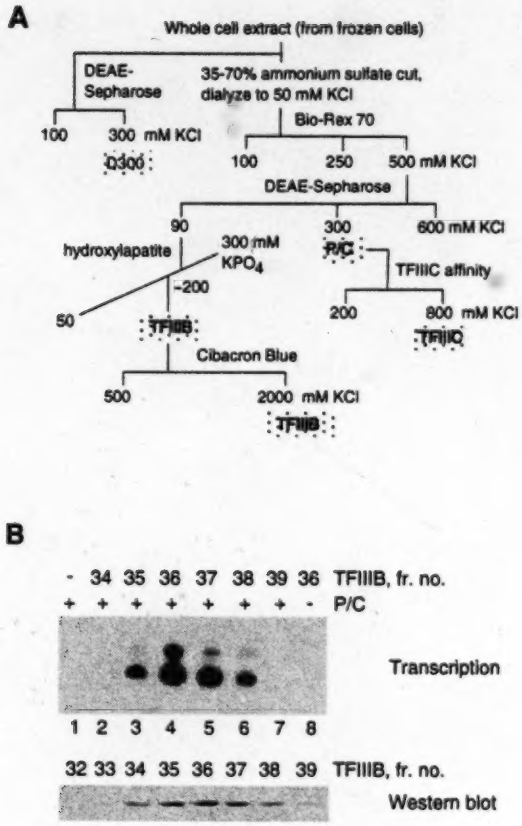

Figure 6-1. Fractionation and functional assay of yeast pol III transcription factors.

A. Scheme for fractionation of pol III transcription factors (adapted from Kassavetis et al. 1995).

B. In vitro reconstitution of pol III transcription (upper panel) by hydroxylapatite fractions of TFIIIB and pol III/TFIIIC (P/C). TBP immunoblot of hydroxylapatite fractions of TFIIIB (lower panel).

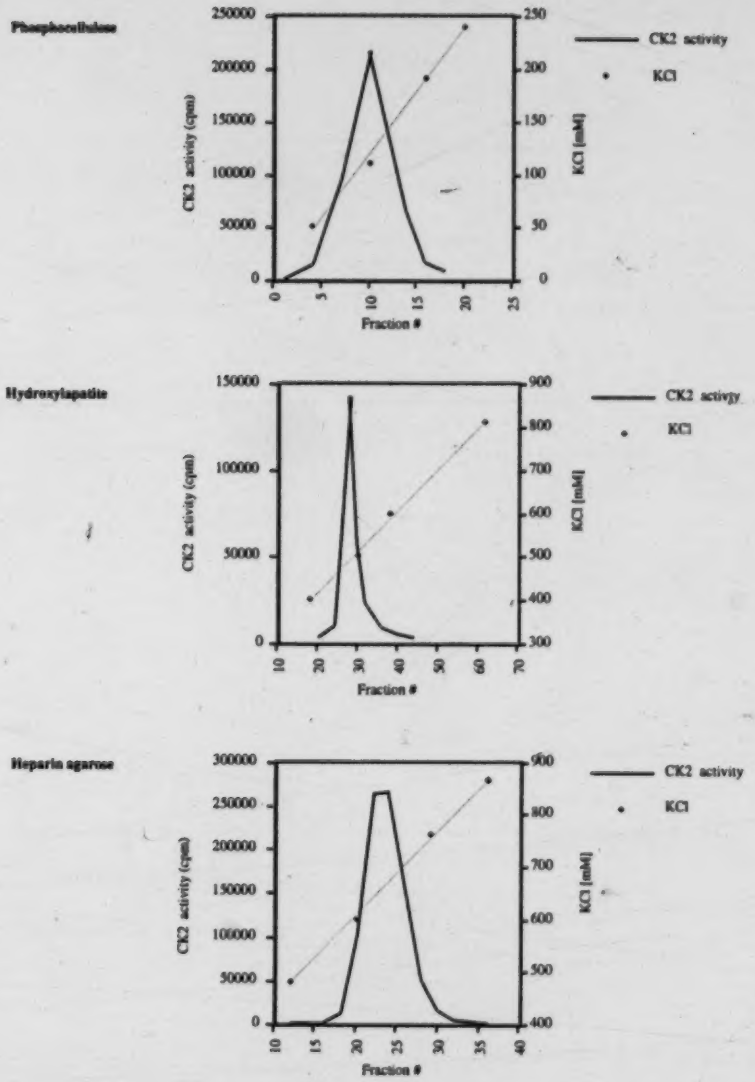

Figure 6-2. Fractionation of yeast CK2. CK2 was purified according to a procedure by Bidwai et al. (1994). Throughout chromatography, the CK2 content of each fraction was determined by phosphorylation of a CK2 substrate peptide. The assays were performed in 25 µl reactions containing 2, 1, and 0.2 µl of each fraction eluted from phosphocellulose, hydroxylapatite, and heparin agarose resins, respectively.

Chapter 7

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